Research Article

Galectin-12 colocalizes with splicing factor-rich speckles and shuttles between the nucleus and cytoplasm in colon cancer cells

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Abstract

Several members of the glycan-binding family of galectins have been linked to colon cancer initiation and progression while the role of galectin-12 in this malignancy is largely unexplored. In previous studies we observed expression of galectin-12 in normal colon epithelium in contrast to its lack of expression in colorectal cancer tissues. In order to gain insight into its molecular function we established a genetically engineered colon cancer model cell line, which facilitates inducible and reconstituted expression of *LGALS12* transgene at physiological levels in an isogenic background. Regulation of transgene expression by doxycycline was confirmed at the transcript- and protein level in a time- and dose-dependent manner for two independent clones. Reconstituted galectin-12 expression did not affect cell morphology and proliferation. Analysis of its subcellular distribution showed that galectin-12 resides in and shuttles between the nucleus and cytoplasm. More detailed analysis by double-immunofluorescence and confocal microscopy revealed colocalization of galectin-12 with splicing-factor rich nuclear speckles, hinting towards a potential role of galectin-12 in pre-mRNA splicing and processing. Therefore, the established model cell line represents a powerful tool to investigate the functional impact of galectin-12 reconstitution on colon cancer tumorigenesis.

Introduction

In recent years, galectins (Gal), a protein family with ß -galactoside binding affinity, have gained increasing attention as important drivers of many different diseases including cancer (Ebrahim *et al.* 2014). Carbohydrate binding is mediated through a conserved carbohydrate recognition domain (CRD) and major ligands are branched N-glycans found on transmembrane proteins promoting the formation of galectin lattices modulating signaling cascades (Wu *et al.* 2011). By translating the sugar code into molecular functions galectins can therefore affect processes such as cell-cycle progression, differentiation, apoptosis, cell-cell interaction and adhesion to the extracellular matrix (Gabius *et al.* 2016, Kaltner *et al.* 2017). In addition, in particular for intracellular functions, also direct glycan -independent galectin-protein interactions have been suggested (Liu & Wang 2002, Wang *et al.* 2004). Thus, maintaining cellular homeostasis requires tight-controlled gene expression levels by various mechanisms and abnormal expression has been linked to tumor initiation and progression including colon cancer (CRC) (Thijssen *et al.* 2015, Timoshenko 2015, Wu *et al.* 2013).

CRC carcinogenesis is associated with an increase in Gal-1, -3 and -7 expression and decrease of Gal-2, -4, -8, -9 and -12 levels thereby contributing to key processes like metastasis formation and immune evasion (Barrow *et al.* 2011, Gopalan *et al.* 2016, Kim *et al.* 2013, Thijssen *et al.* 2015). For instance, Gal-3 binds to oncofetal Thomsen-Friedenreich antigen on the transmembrane protein MUC1 promoting its cell surface polarization resulting in enhanced adhesion of cancer cells to the vascular endothelium (Mori *et al.* 2015). On the contrary, Gal-4 exerts a tumor suppressive function by induction of cell cycle arrest and differentiation (Michalak *et al.* 2016, Satelli *et al.* 2011). Compared to Gal-1 and -3 which are the most extensively studied members of this family, the functional role of Gal-12 has only rarely been investigated. Gal-12, encoded by the *LGALS12* gene, is a bivalent tan-

dem-repeat type galectin, which consists of 2 CRDs joined by a short linker sequence. Lactose binding affinity has been confirmed, though the C-terminal CRD lacks several of the key amino acid residues known to be responsible for carbohydrate interaction and are conserved among the other members of the family (Yang *et al.* 2001). Most tissues and cell lines are characterized by low mRNA levels of *LGALS12*, however strong expression has been observed in peripheral blood and myeloid cells as well as adipocytes (Yang *et al.* 2001). Gal-12 expression can also be triggered by different conditions such as oxidative stress and differentiation (Vinnai *et al.* 2017, Yang *et al.* 2004). The vacuolar protein VPS13C has been identified as an interacting partner of Gal-12 and loss of VPS13C expression results in rapid degradation of Gal-12 through the lysosomal pathway (Yang *et al.* 2016). While the biological role of Gal-12 has been mainly studied in adipocytes, the function in other cell types is largely unexplored. In adipocytes abrogation of Gal-12 severely impairs cellular differentiation and adipogenic signaling by downregulation of critical transcription factors such as CCAAT/enhancer-binding protein (Yang *et al.* 2004). Ablation of Gal-12 in mice increases mitochondrial respiration, reduces inflammation and adiposity suggesting involvement in metabolic disorders (Yang *et al.* 2011).There is also growing evidence that Gal-12 could be an important modulator in controlling tumor growth and progression. Ectopic expression can affect proliferation and possesses apoptosis-inducing activity in tumor cells of different origin (Hotta *et al.* 2001, Yang *et al.*2001). Furthermore, downregulation of *LGALS12* mRNA has been observed in several tumor entities and correlates with lower overall survival as well as enhanced prevalence of tumor recurrence and lymphovascular invasion (El Leithy *et al.* 2015, Gopalan *et al.* 2016, Laderach *et al.* 2013, Salajegheh *et al.* 2014).

We have previously reported that loss of Gal-12 expression is associated with promotor hypermethylation in CRC cells and *de novo* expression can be triggered upon induction of differentiation by butyrate, a short chain fatty acid involved in maintaining intestinal homeostasis (Katzenmaier *et al.* 2017, Katzenmaier *et al.* 2014). In accordance with the data obtained from our *in vitro* study Gal-12 was found to be constitutively expressed in the differentiated healthy colon epithelium but significantly decreased in corresponding tumor tissue (Katzenmaier *et al.* 2017). Galectins are known as important modulators of cell growth and differentiation. However, the extent to which silencing of Gal-12 contributes to growth advantages of colon tumor cells and its biological function in CRC remains still unclear. In the present study we therefore established a doxycycline-inducible Gal-12 expressing CRC cell line, which enables to study the impact of Gal-12 reconstitution on CRC tumorigenesis. Time- and dose-dependent Gal-12 expression as well as the intracellular distribution pattern were examined. In addition, we demonstrate for the first time colocalization of Gal-12 with splicing speckles in the nucleus, suggesting involvement in pre-mRNA splicing and processing.

Materials and Methods

Plasmids

The retroviral expression plasmid S2F-cLM2CG-FRT3 contains a tet-controlled bidirectional transcription unit, which facilitates Doxycycline (Dox)-dependent expression of the two reporter genes firefly *luciferase* and red fluorescent protein *mCherry* (Weidenfeld *et al.* 2009). Additionally, the expression cassette is flanked by two recombination sites, a mutated F3 and wildtype F site, that can be recognized by Flpo-recombinase (Schlake & Bode 1994). For generation of the S2F-cLM2CG-FRT3-Gal12 construct the *Eco*R 1/*Not* 1 *mCherry* fragment was replaced by human galectin-12 (Gal-12) full-length cDNA (NM_033101) derived from SW707 cells, cultured in the presence of 1 mM NaBut for 7 days, resulting in *de novo* expression of *LGALS12* at transcript level. cDNA was amplified using primers carrying *Eco*R 1 and *Not* 1 restriction sites as well as a N-terminal Flag-Tag sequence. Verification of the amplified *LGALS12* sequence, with or without integrated Flag-Tag, was confirmed by sequencing. The plasmid pCAGGS-Flpo-IRES-Puro encodes for Flpo-recombinase, which was used for recombinase mediated cassette exchange (RMCE).

Cell Lines and Culture Conditions

Cell lines were either cultured in DMEM-F12 (PAA, Cölbe, Germany) (HCT116-Tet-On, HCT116-HygTK, HCT116-Gal12) or RPMI1640 (PAA, SW707 cell line) supplemented with 10% FBS (PAA), 1% Penicillin (PAA) and 1% Streptomycin (PAA) at 37 °C in a humidified atmosphere containing 5 % CO_2. HCT116-Tet-On cells are stably transfected with a construct facilitating constitutive expression of a reverse transactivator (rTA) and EGFP. (*Welmann et al.* 2006). HCT116-HygTK cells additionally contain a hygromycin B thymidine kinase (HygTK) expression cassette flanked by two recombination sites (F/F3) conferring hygromycin resistance (Lee *et al.* 2013). Cells were regularly checked for mycoplasm contamination using the Mycoplasma Detection Kit from Minerva Biolabs (Berlin, Germany). For reconstitution of Gal-12 ex-

pression, cells were either grown in the presence or absence of 1 µg/ml Dox (Sigma-Aldrich, Taufkirchen, Germany) for 48 h. To investigate time- and dose-dependent inducible expression of *LGALS12*, cells were grown in the presence or absence of 1 µg/ml Dox for 30 min up to 96 h and incubated with different concentrations of Dox (0.005-1 µg/ml) for 48 h respectively.

Generation of HCT116-Gal12 Cells

HCT116-Gal12 cells were generated by using the master cell line HCT116-HygTK, which is hygromycin resistant but sensitive to ganciclovir. 10^7 cells were electroporated with 3 µg of S2F-cLM2CG-FRT3-Gal12 and 1 µg of pCAGGS-Flpo-IRES-Puro plasmid using the AmaxaCell Line Nucleofactor Kit V (Lonza, Basel, Switzerland) according to the manufactures instructions. Upon RMCE the integrated HygTK expression cassette was replaced by an expression cassette encoding for luciferase and Gal-12. After 24 h 1.5 µg/ml puromycin (Sigma-Aldrich) was added to the growth medium for 36 h followed by selection with 40 µM Ganciclovir (Roche, Mannheim, Germany) for at least 2 weeks. Integration of the expression cassette in individual clones was confirmed by sequencing and the inducibility was determined by performing quantitative real-time PCR and western blot analysis as well as measuring luciferase activity

Reverse Transcriptase PCR (RT-PCR) and Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted employing RNeasy Mini Kit (Quiagen, Hilden, Germany) following the manual procedure. 1 µg of total RNA was subsequently transcribed into cDNA as described previously (Katzenmaier *et al.* 2014). Human full-length *LGALS12* cDNA of SW707 cells was amplified with primer carrying an *EcoR* 1 and *Not* 1 restriction site as well as a Flag-Tag sequence under following cycle conditions: activation of DNA polymerase (Phusion High Fidelity DNA Polymerase; Biocat GmbH, Heidelberg; Germany) at 98 °C for 30 sec followed by 40 cycles at 98 °C for 10 sec and 72 °C for 40 sec with a final extension at 72 °C for 10 min. For qRT-PCR analysis cDNA was diluted 1:10 and analyzed in triplicates using the StepOne Plus thermo cycler (Applied Biosystems, Thermo Fisher Scientific, Waltham, USA). The cycling program was as followed: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. Gene expression was normalized to expression of the housekeeping gene *Hydroxymethylbilan-Synthase (HMBS)*. Data quantification was performed by applying StepOne Software v2.1. Fold change of expression was determined according to the ΔΔCt method (Livak & Schmittgen 2001). Primer sequences are given in Table S1.

DNA Sequence Analysis

Genomic DNA of HCT116-Gal12 cells was extracted using DNeasy Blood and Tissue Kit (Quiagen) following the manufacturer's instructions. Sequencing of the amplified gDNA and cDNA of *LGALS12* was performed with the Big Dye Terminator v1.1 sequencing kit (Thermo Fisher Scientific) on an ABI3100 genetic analyzer. Sequencing primers are indicated in Table S1.

Luciferase Assay

Luciferase activity was determined in duplicates by the Luciferase Assay System (Promega Mannheim, Germany) according to the manufacturer's protocol. Relative light units were normalized to protein concentration measured by Bradford assay (Bio Rad, Munich, Germany).

Western Blot Analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1 mM $CaCl_2$, 0.01 mM $MgCl_2$, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.4) supplemented with protease inhibitors (cOmplete mini, Roche, Basel, Switzerland) and sonicated for 30 sec on ice. After incubation on a rotator at 4 °C for 1 h, samples were centrifuged at 21.000 g and 4 °C for 20 min. Subsequently, the supernatant was collected for western blot analysis and protein concentration was determined by Bradford assay. Separation and blotting of proteins on a nitrocellulose membrane was carried out as previously described (Katzenmaier *et al.* 2014). Briefly, after blocking the membrane for at least 30 min at room temperature (RT) in 5% skim milk/TBST (20 mM Tris-HCl pH 7.5, 0.5 M NaCl and 0.1% Tween-20) or 5% BSA/TBST, the following primary antibodies were used in blocking solution: rabbit anti-Flag (F7425, 1:500, 5% milk/TBST, overnight 4 °C, Sigma-Aldrich), mouse anti-β-Actin (clone C4, 1:2.000, 5% milk/TBST, overnight 4°C, MP Biomedicals, Solon, USA), mouse anti-GAPDH (mAbcam9484, 1:1.000, 5% BSA/TBST, 1 h RT, Abcam, Cambridge, GB) and rabbit anti-Lamin B1 (clone D9V6H, 1:1.000, 5% milk/TBST, overnight 4 °C, Cell Signaling Technology, Danvers, USA). After the incubation period membranes were washed thrice with TBST (10 min each at RT) and incubated either with HRP-labeled anti-rabbit antibody (1:2.000, 5% milk/TBST, Promega) or anti-mouse-IgG antibody (1:5.000, 5% milk/TBST, GE Healthcare, Munich, Germany) for 1 h at RT. Visualization of the proteins was performed by chemiluminescence.

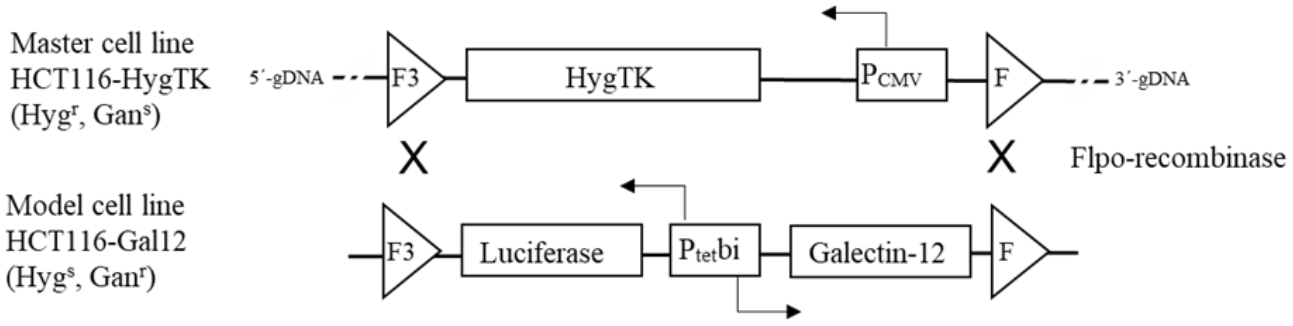

Figure 1. Generation of HCT116-Gal12 cells by recombinase-mediated cassette exchange (RMCE). The master cell line HCT116-HygTK was used for RMCE (Lee *et al.* 2013). This cell line carries an expression cassette encoding for the fusion protein hygromycin B and thymidine kinase flanked by 2 Flpo-recombinase recognition sites, a wildtype (F) and mutant one (F3). These recombination sites facilitating the exchange of the expression cassette with a luciferase-Gal12 expression cassette by RMCE resulting in the model cell line HCT116-Gal12 (adapted from Lee *et al.*2013).

Density gradient centrifugation

$2.4x10^6$ HCT116-Gal12 cells were grown on T175 flasks in the presence or absence of 1 µg/ml Dox for 48 h. Subsequently, medium was removed and the cell layer was washed twice with ice cold PBS, scraped and centrifuged for 3 min at 800 g and 4 °C. Cell pellets of 2 flasks were collected, resuspended in 1 ml cell lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, pH 7.4) and applied on top of 3 ml sucrose buffer (0,7 M Sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, 2 mM EDTA, 0.5 mM EGTA, 14 mM β-Mercaptoethanol, 0.1% Triton-X-100, pH 7.5). In addition, trypan blue staining was performed to control for cell lysis. After centrifugation for 10 min at 1.300 g and 4 °C the cytoplasmic fraction was harvested from the top of the sucrose buffer and the nuclei, forming a cell pellet at the bottom of the tube, were resuspended in 500 µl RIPA cell lysis buffer supplemented with protease inhibitors. Protein concentration was determined by Bradford assay.

Immunofluorescence staining and Imaging

10^4 cells were grown on cover slips in the presence or absence of 1 µg/ml Dox for 48 h. Afterwards cells were washed once with PBS and fixed with ice cold acetone and methanol (8 min each, -20°C). Subsequently, cells were washed twice with PBS for 5 min, blocked with 10% BSA/PBS for 30 min (RT) and incubated with the following primary antibodies in 3% BSA/PBS in a humid chamber at 4 °C overnight: rabbit anti-DYKDDDDK Tag (clone D6W5B, 1:50, Cell Signaling) and mouse anti-SC35 (ab11826, 1:400, Abcam). Samples were washed thrice with PBS for 5 min and incubated with the listed secondary antibodies in 3% BSA/PBS for 1 h at RT: Alexa Fluor 488 labeled anti-mouse IgG (A21121, 1:800, Thermo Fisher Scientific) and Alexa Fluor 594 labeled anti-rabbit IgG (ThA21442, 1:200, Thermo Fisher Scientific). After 3 washing steps with PBS (5 min each) counterstaining of the nuclei with DAPI (Thermo Fischer Scientific) was carried out in 3% BSA/PBS for 10 min. Finally, cells were washed twice with PBS (5 min each) and embedded using DAKO Fluorescence Mounting Medium (DAKO, Hamburg, Germany). For colocalization studies both primary as well as secondary antibodies were applied at the same time. Immunofluorescence analysis was carried out using an Olympus AX 70 (40 x magnification) microscope. For colocalization studies confocal laser scanning microscopy was conducted equipped with a Plan-Apochromat 63x/1.40 Oil DIC objective, a UV diode 405 nm (DAPI), an Argon 488 nm (FITC) and Helium-Neon 594 nm (TRITC) laser as well as reflected light photomultiplier tubes. Image acquisition and processing was performed using Leica LAS AF and ImageJ software (Schindelin *et al.* 2012). Background was substracted with constant settings using ImageJ's Rolling ball background subtraction.

MTT Assay

Cells were grown on 96 well plates in the presence or absence of 1µg/ml Dox for up to 96 h. Proliferation was assessed by reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium to Formazan using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, Mannheim, Germany) according to the manufacturer`s protocol.

Enzyme activity for Alkaline Phosphatase

As a marker of cellular differentiation alkaline phosphatase activity was assessed in HCT116-Gal12 #5+F cells treated with or without 1µg/ml Dox for 5 days as described previously (Katzenmaier *et al.* 2014). One unit was defined as the amount of enzyme that hydrolyzes 1 µmol p-nitrophenylphosphate into p-

nitrophenolate per minute. Protein concentration was determined by the method of Lowry.

Results

Generation of a Colorectal Cancer (CRC) Model Cell Line with Doxycyline-inducible Gal-12 Expression

In order to investigate the functional consequence of Gal-12 expression on CRC tumorigenesis we established a Gal-12 inducible CRC model cell line, which is a derivate of the CRC cell line HCT116. HCT116 cells represent a suitable model to assess the functional role of Gal-12 because they lack expression of the *LGALS12* gene similar to what has been observed in primary CRC tumors. Since Gal-12 has been reported to confer anti-proliferative and pro-apoptotic functions in different cell types a doxycycline-inducible (Tet-on) gene expression system was chosen to account for potential growth suppressive functions in colorectal cancer cells (Wan *et al.* 2018). For generation of HCT116-Gal12 cells the master cell line HCT116-HygTK was used, which constitutively expresses a dox-regulated reverse transactivator (rTA) and additionally contains an expression cassette flanked by 2 Flpo-recombinase recognition sites conferring hygromycin resistance. Two independent clones of this master cell line have been established in our lab previously, differing in the integration site of the expression cassette, which is either localized on chromosome 1 (C1orf159) for clone #5 and on chromosome 5 (*ALDH1L1* gene, silenced by methylation in HCT116 cells (Lee *et al.* 2013, Oleinik & Krupenko 2011)) for clone #22. The luciferase-Gal12 expression cassette was inserted directly at these defined loci by recombinase mediated cassette exchange resulting in HCT116-Gal12 #5 and #22 cells (Figure 1). In addition, and due to the lack of Gal-12 specific antibodies, clones with an integrated Flag-tag at the N-terminus of Gal-12 were also generated (+F = with Flag, -F=without Flag). The established cell line therefore facilitates for the first time the investigation of the biological relevance of Gal-12 on CRC tumorigenesis.

Characterization of HCT116-Gal12 cells

Next, two independent clones of our established model cell line were characterized in more detail. Dox treatment with 1 µg/ml over 48 h resulted in an increase in luciferase activity (#5-F: 78x; #22-F: 345x) (Figure 2A) as well as strong induction of the *LGALS12* transcript by qRT-PCR (#5-F: 154, #22-F: 110x) (Figure 2B) confirming the inducibility of the construct.

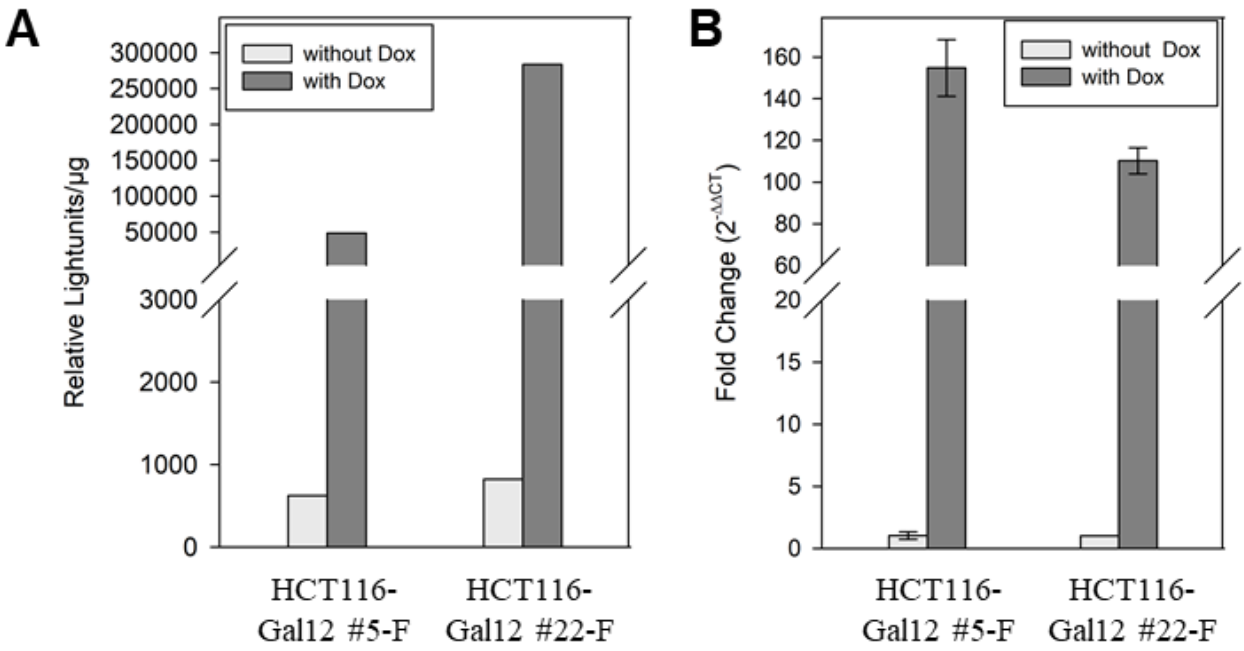

Figure 2. Induction of luciferase activity and *LGALS12* mRNA expression by treatment with Doxycycline. HCT116-Gal12 #5-F and #22-F cells were cultured in the presence or absence of 1 µg/ml Dox for 48 h. Treatment resulted in strong increase of luciferase activity (A) as well as induction of *LGALS12* mRNA expression (B, qRT-PCR) in 2 independent clones, which differ in the integration site of the expression cassette. Values represent the mean of three independent experiments ± SD. Data are shown for clone HCT116-Gal12 #5-F and #22-F but also apply to clones expressing the Flag-tagged counterpart (not shown).

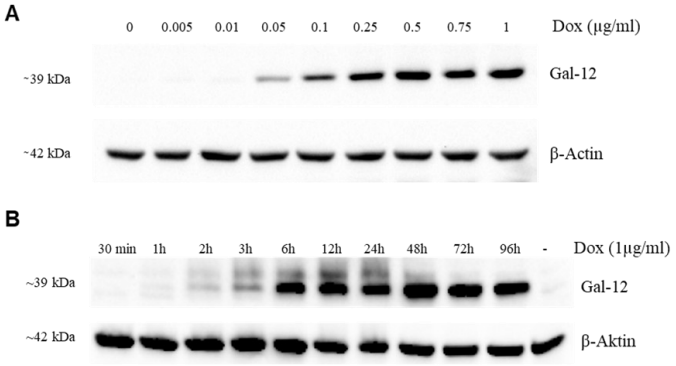

Figure 3. Dose- and time-dependent induction of Gal-12 protein expression. HCT116-Gal12 #5+F cells were cultured either in the presence or absence of 0.1-1 µg/ml Dox for 48 h (A, dose-response) or 1 µg/ml Dox for up to 96 h (B, time-course) before Western blot analysis was performed. The strongest induction of Gal-12 expression was achieved with 1 µg/ml Dox over 48 h. In the absence of Dox no Gal-12 protein was detectable. Data are shown for clone HCT116-Gal12 #5+F but also apply to clone HCT116 -Gal12 #22+F (not shown).

Apart from the induction of the luciferase marker protein we also examined the Dox-inducible expression of the Gal-12 protein by western blot analysis. Due to the lack of Gal-12 specific antibodies, two independent clones expressing N-terminal Flag-tagged Gal-12 fusion protein were used for these experiments (HCT116-Gal12 #5+F; HCT116-Gal12 #22+F). Dose-dependent expression analysis revealed the highest induction of Gal-12 protein upon treatment with 1 µg/ml Dox (Figure 3A). When we performed a time-course analysis, Gal-12 expression was detectable already after 2 h and protein levels reached a peak within 48 h (Figure 3B). Even after 96 h, a high level of Gal-12 protein was observed which facilitates the investigation of short and long-term effects of Gal-12 on CRC carcinogenesis. Remarkably, no Gal-12 protein was detectable in cells cultured without Dox confirming the lack of leakiness of the applied Tet-system. Since treatment with 1 µg/ml Dox for 48 h resulted in the strongest induction (Figure 3A) this concentration was chosen as a standard culture condition for all subsequent experiments.

Inconsistent with the published literature (Wan *et al.* 2018) reactivation of Gal-12 expression did not induce any alterations in cell cycle progression or differentiation status (Figure S3 A,B) of the model cell line. Also, no major differences were observed between both clones. Based on these results, our genetically engineered HCT116-Gal12 cells provide a useful model system to examine the cellular and molecular function of Gal-12 in more detail.

Cell cycle-dependent subcellular distribution of Gal -12

Subcellular location of proteins can provide important clues about their potential cellular functions. Several studies have shown that galectins can be found in many distinct subcellular compartments as well as on the cell surface and in the extracellular milieu (Santos *et al.* 2016, Takenaka *et al.* 2004). However, the subcellular localization of Gal-12 in CRC cells has not been explored yet. Therefore, we examined the subcellular distribution profile of Gal-12 in HCT116-Gal12 #5+F cells by cell fractionation and density gradient centrifugation. The purity of the separated fractions was verified by antibodies directed against well-

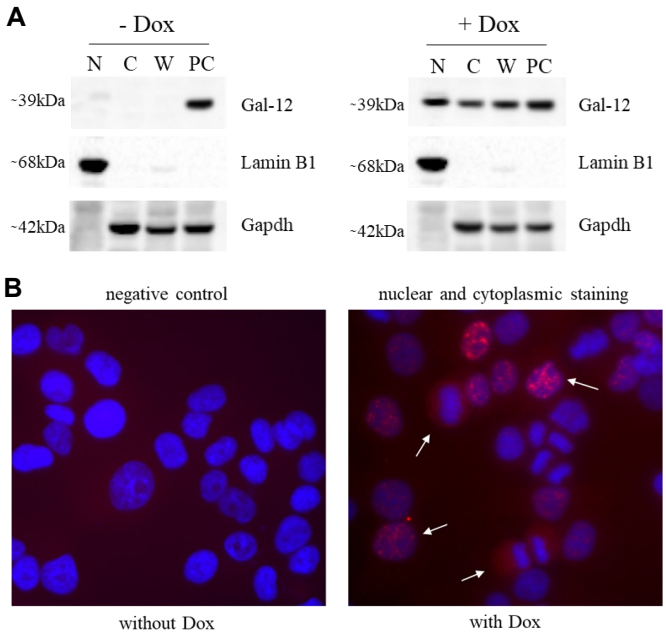

Figure 4. Subcellular distribution of Gal-12. A) Subcellular fractions of HCT116-Gal12 #5+F cells, cultured for 48 h in the presence (+) or absence of (-) of 1 µg/ml Dox, were obtained by density gradient centrifugation. The purity of each fraction was confirmed by detection of nuclear Lamin B1 and cytoplasmic GAPDH protein using western blot analysis. Gal-12 was present in both fractions of Dox-treated cells but was absent in untreated cells. HCT116-Gal12 cells, previously documented to be positive for Gal-12, served as a positive control (N =nuclear, C = cytoplasmic, W= whole cell lysate, PC= positive control) B). For immunofluorescence staining cells were grown on coverslips under the same culture conditions as described above. After 48 h cells were fixed and the nuclei were counterstained with DAPI (blue). Immunostaining of Gal-12 was performed as described in detail in Material and Methods using a Flag-antibody and an Alexa Fluor 594 labeled secondary antibody. Subcellular localization of Gal-12 (pink to red), indicated by white arrows, is shown for cells in the interphase and various stages of mitosis. Cells cultured in the absence of Dox served as a negative control (400x magnification).

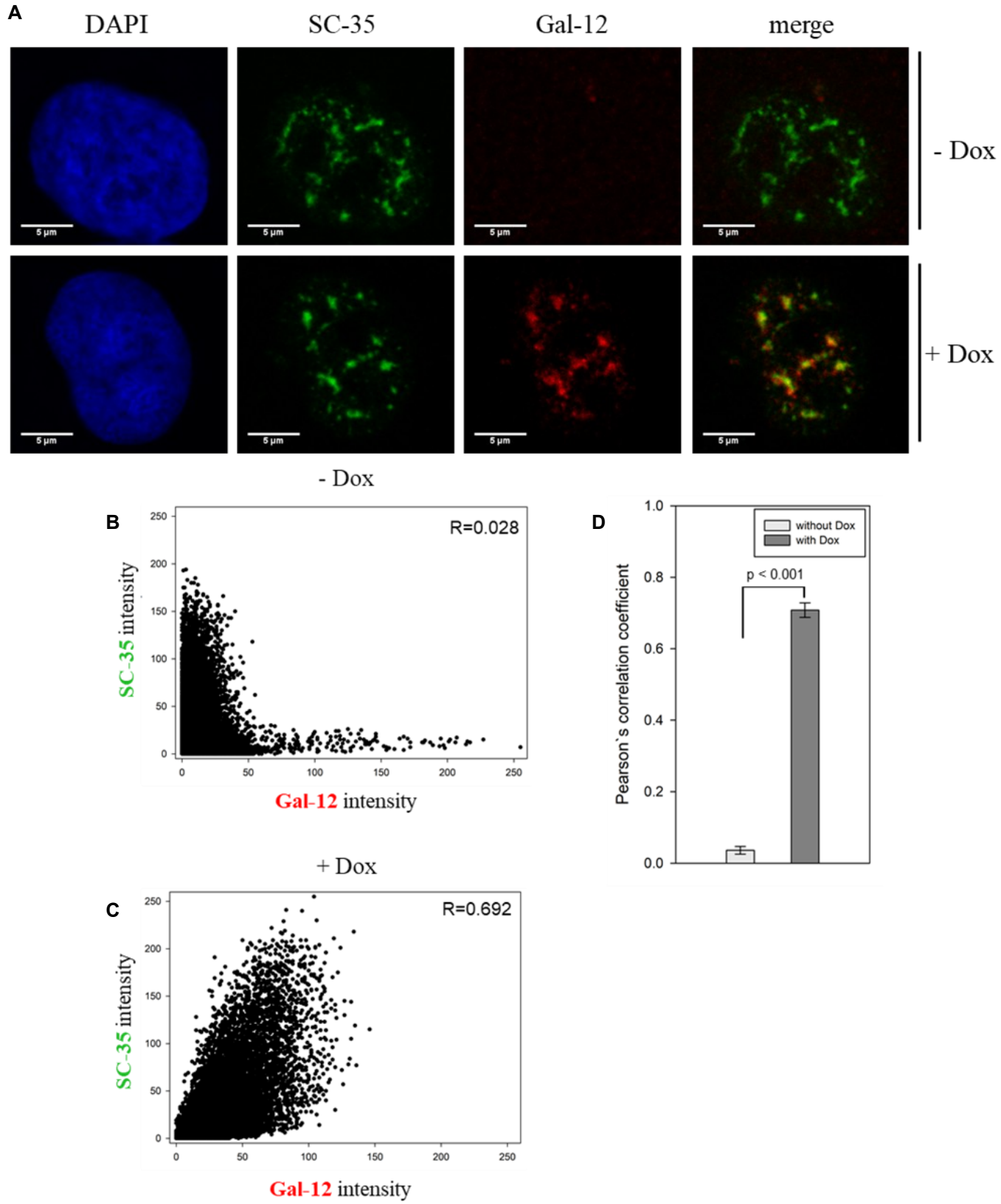

Figure 5. Colocalization analysis of Gal-12 with SC-35. Double immunofluorescence staining was performed to investigate colocalization of Gal-12 (Alexa Fluor 598) and SC-35 (Alexa Fluor 488) by confocal laser scanning microscopy (A). Cell nuclei were counterstained with DAPI. Gal-12 protein is shown in red whereas SC-35 is marked with green fluorescence. Overlay of the fluorescence signals confirms colocalization of both proteins (yellow and orange) in nuclear speckles of HCT116-Gal12 #5+F cells cultured in the presence of 1 µg/ml Dox for 48 h. Corresponding scatter plots of untreated (B) and treated cells (C) represent the colocalization tendency between both proteins. The extent of colocalization was further assessed by calculation of Pearson`s correlation coefficients (R). Values reflect the mean of three analyzed cells in one visual field (D) from each culture condition ± SD ($p < 0.001$, t-test). Images and scatter plots of the additionally analyzed cells are shown in Supplementary Material Fig. S1 and S2.

established marker proteins of the nucleus (Lamin B1) and cytosol (GAPDH). As expected, Lamin B1 protein was enriched in the nuclear fraction whereas the glycolytic enzyme GAPDH was found to be present in the cytoplasmic fraction only (Figure 4A). Interestingly, in Dox-treated HCT116-Gal12 #5+F cells, Gal-12 occurred in both subcellular protein fractions but remained undetectable in untreated cells. To investigate the spatio-temporal subcellular distribution pattern of Gal-12 more in detail immunofluorescence staining was performed using a Flag-specific antibody. Compared to the results obtained by density gradient centrifugation, Gal-12 was found to reside predominantly in the nucleus recognized as a speckled staining pattern sparing the nucleoli (Figure 4B). A more detailed analysis of the immunofluorescence images revealed that the subcellular distribution of Gal-12 seemed to vary in a cell cycle-dependent manner. Thus, looking at single Dox-treated cells at various stages of mitosis, the speckled staining pattern disappeared and Gal-12 was found to be translocated to and homogeneously distributed in the cytoplasm without any nuclear staining (Figure 4B). Only a weak background fluorescence signal was visible in untreated HCT116-Gal12 #5+F cells confirming the specificity of the applied antibody. These results indicate, that the subcellular localization of Gal-12 protein can vary significantly, and is potentially affected in a cell cycle-dependent manner.

Gal-12 colocalizes to splicing-factor rich nuclear speckles

The observed speckle-like distribution pattern of Gal-12 in the nucleoplasm, sparing the nucleoli, is strikingly reminiscent of that described for splicing-factor rich nuclear speckles, a subcellular compartment where splicing factors are stored or reassembled. Thus, in the next step colocalization of Gal-12 with serine/arginine-rich splicing factor 2 (SC-35), a commonly used marker for nuclear speckles, was assessed by applying confocal laser scanning microscopy. Double immunofluorescence staining of SC-35 and Gal-12 revealed strong colocalization of both proteins in the nuclei of Dox treated HCT116-Gal12 #5+F cells, represented by the yellow and orange color in the overlay of both images (Figure 5A, S1 C,D). The degree of colocalization was confirmed by analyzing the spatial correlation of the fluorescence signals obtained for each protein as indicated by quantitative scatter plots (Figure 5 B,C). Scatter plots of additionally analyzed cells are depicted in Supplementary Materials (Figure S2 A-D). The observed average Pearson correlation coefficient of 0.7 of 3 analyzed cells in one visual field indicates an overlap of both fluorescence signals of approximately 70 % (Figure 5D). The spatial distribution and expression of SC-35 remained unaffected by Dox treatment (Figure 5A, S1 A,B). Furthermore, only a weak background fluorescence signal intensity was obtained in untreated cells after incubation with flag-specific antibody, confirming the specificity of the observed staining pattern (Figure 5A, S1 A,B). These data for the first time demonstrate colocalization of Gal-12 with nuclear speckles, suggesting involvement in pre-mRNA splicing and processing, thereby pointing to a new functional role of this rarely studied member of the galectin family.

Discussion

As translators of the sugar code, galectins have emerged as important modulators of tumor development, progression and metastasis (André *et al.* 2015). Their biological function and mode of reprogramming during tumorigenesis has been well established for several members of this protein family (Dings *et al.* 2018). Still, for some of them like Gal-12, their function in human malignancies, especially in colorectal carcinogenesis, has only rarely been investigated. In the present study we have established a CRC model cell line genetically engineered to enable inducible and reconstituted expression of a *LGALS12* transgene. This model system has several major advantages. First and most importantly, this model cell line carries a single copy of the *LGALS12* transgene integrated at a defined genomic locus in an isogenic background. Second, the use of independent clones differing in the genomic integration site of the *LGALS12* transgene expression cassette enables to exclude any locus and clone-specific effects. Third, transgene expression can be regulated by doxycycline in a dose-dependent and fully reversible manner, thereby enabling physiological Gal-12 expression levels, and allowing the investigation of short and long-term Gal-12-mediated effects on CRC tumorigenesis. Fourth, this model system can be applied to multi-omics approaches such as transcriptome, proteome and secretome analysis and can also be used in xenograft studies thereby facilitating detailed functional analysis at the molecular level. Finally, this inducible expression system represents a versatile tool that can be easily adapted to any other galectin or gene of interest. When using this model cell line it should, however, be recalled that this reconstituted Gal-12-expression system reflects the inverse situation of primary CRC tumors which are characterized by loss of *LGALS12* expression (Katzenmaier *et al.* 2017).

When we examined the molecular and cellular consequences of reconstituted *LGALS12* transgene expression in this model system, none of the two inde-

pendent clones showed any alterations of cell size, shape or morphology under optimal induction conditions. Likewise, reconstituted expression of Gal-12 did not affect cell growth and differentiation of these cultured cells. In our previous study Gal-12 was found to be *de novo* expressed upon induction of differentiation of CRC cells (Katzenmaier *et al.* 2014). Studies in HeLa cells and adipocytes revealed that ectopic expression of Gal-12 leads to growth arrest and differentiation (Yang *et al.* 2001*,* 2004). Several explanations might account for these apparently opposing results. First, Gal-12 might not primarily drive differentiation of CRC cells but rather is involved in maintaining cellular homeostasis of cells with a higher degree of differentiation. Second, appropriate i.e. just right levels of Gal-12 expression might be required for specific biological functions. Compared to the literature using cell lines with multiple integration sites of the *LGALS12* transgene our model system carries just one copy reflecting more likely physiological levels of Gal-12 protein (Yang *et al.* 2001). Such dose-dependent effects have been already reported for Gal-1 causing biphasic modulations of cell growth (Adams *et al.* 1996). Furthermore, alternative splicing gives rise to multiple isoforms of *LGALS12* differing in the length of the linker connecting both CRDs. Since tissue dependent effects of Gal-12 on cellular differentiation have been reported, it seems likely that expression of tissue-specific isoforms additionally contribute to functional diversity (Huiting *et al.* 2016, Wan *et al.* 2016). Different roles of Gal-9 isoforms in Lovo cells and endothelial cells have been demonstrated (Aanhane *et al.* 2018, Zhang *et al.* 2009). While Gal-9N and Gal-9M stimulate tube formation, Gal-9C inhibits sprouting. However, it remains to be elucidated whether this also applies for Gal-12 variants.

Our study also provided insights into the subcellular localization of Gal-12 in colorectal cancer cells. Based on immunofluorescence and density gradient centrifugation analysis we demonstrate that Gal-12 resides in and shuttles between the nucleus and cytoplasm of colorectal cancer cells. This finding in colorectal cancer cells compares very well with studies of other cell types including sebocytes and adipocytes (Harrison *et al.* 2007). In adipocytes Gal-12 was additionally detected in the mitochondrial fraction and localized to lipid droplets (Hotta *et al.* 2001, Yang *et al.* 2011). Since we did not extract the mitochondrial fraction from the cytosol we cannot exclude the presence of Gal-12 in mitochondria of CRC cells. However, compared to the other studies, our data reveal for the first time nucleocytoplasmic shuttling of Gal-12 in a cell cycle dependent manner. Shuttling between both subcellular compartments has been only demonstrated for Gal-3 promoting its antiapoptotic activity and drug resistance (Arnoys *et al.* 2015, Takenaka *et al.* 2004, Yu *et al.* 2002). Several members of the galectin family have been detected in the nucleus including Gal-1, -2, -3 and -7 (Dvoránková *et al.* 2008, Kodet *et al.* 2011). Although the precise nuclear transport pathway is still unknown distinct mechanisms have been proposed including passive diffusion and active transport (Funasaka *et al.* 2014, Nakahara *et al.* 2006). For instance, an importin-α/β-mediated nuclear localization signal and an exportin-1-mediated nuclear export have been confirmed for Gal-3 (Davidson *et al.* 2006, Nakahara *et al.* 2006, Nakahara & Raz 2007). In addition, posttranslational modifications have been shown to influence the subcellular distribution of galectins. While non-phosphorylated Gal-3 is exclusively found in the nucleus phosphorylation on Ser6 drives its nuclear export resulting in accumulation in the cytoplasm (Takenaka *et al.* 2004). The polypeptide sequence of Gal-12 contains several potential phosphorylation sites. It thus seems warranted to investigate in more detail whether phosphorylation acts as a switch in the subcellular distribution of Gal-12. Galectins are synthesized on cytosolic ribosomes lacking a classical signal sequence usually characteristic for secreted proteins (Leffler *et al.* 2002). Strikingly, they are secreted by a non-classical pathway acting on the cell surface or extracellular milieu thereby contributing to different processes such as cell-cell adhesion and adhesion of tumor cells to the vascular endothelium (Hughes 1999, Ilmer *et al.* 2016, Mori *et al.* 2015, Nobumoto *et al.* 2008, Seelenmeyer *et al.* 2005). Compared to other members of the galectin family, secretion of Gal-12 has not been demonstrated yet. Since it was predominantly localized in the nucleus and cytosol of the model cell line it seems likely that it mainly executes intracellular functions. However, changes in the intra- and extracellular distribution of other galectins have been linked to tumor growth and progression (Califice *et al.* 2004, Ilmer *et al.* 2016, Straube *et al.* 2011). Due to the spatial distribution of Gal-12 observed in the present study the question arises, whether intra- and extracellular distribution is affected upon disease progression and if this correlates with clinical features such as differentiation. In this context, identification of compartment-specific Gal-12 interacting proteins should represent a promising approach to understand how such interactions determine the biology of normal colon epithelium and how specific interference might contribute to colorectal tumorigenesis.

One of the most striking findings of this study was the observed dotted pattern of Gal-12 in the nucleus that spared the nucleoli and was quite reminiscent of that of nuclear speckles. Nuclear speckles, also

known as SC-35 enriched splicing speckles, appear as 20-50 irregular shaped dots in the nucleoplasm and are storage and assembly sites for the splicing machinery in the nucleus (Shpargel & Matera 2005). Alternative splicing contributes to the complexity of gene expression and plays an important role in processes such as cellular homeostasis and differentiation. Changes in this defined process can lead to interruption of protein-protein interactions and expression of distinct and novel protein isoforms driving pathogenic conditions such as cancer (Yamaguchi *et al.* 1996). The splicing machinery is build up in a sequential manner by recruitment of several small nuclear ribonucleoproteins (snRNPs) as well as splicing factors such as SC-35 to pre-mRNA (Wahl *et al.* 2009). SC-35 is required for the first step of the splicing reaction by mediating the interaction between the 5´and 3´splice site (Fu & Maniatis 1992). Performing double immunofluorescence staining we demonstrate for the first time colocalization of Gal-12 with SC35 positive nuclear speckles suggesting involvement in pre-mRNA splicing and processing. Colocalization of Gal-1 and -3 with nuclear speckles has been demonstrated in HeLa cells (Dagher *et al.* 1995, Vyakarnam *et al.* 1997). Both galectins are known to act as important pre-mRNA splicing factors and depletion from nuclear extracts reduces splicing activity (Dagher *et al.* 1995, Vyakarnam *et al.* 1997). Immunoprecipitation analysis has further confirmed binding of Gal-3 to Gemin-4 and heterogenous nuclear ribonucleoproteins (hnRNPs), which are involved in snRNP and spliceosome assembly (Fritsch *et al.* 2016, Park *et al.* 2001, Sphargel & Matera 2005). Hence, modulating alternative splicing may be a common and important role of galectins contributing to functional diversity of the cellular proteome.

Although not demonstrated experimentally in the present study, it is tempting to speculate, that Gal-12 might represent a novel pre-mRNA splicing regulator. Galectins preferentially bind to N-acetyllactosamine extensions in N- and O-glycans via the conserved CRD. Although glycosylated proteins are mainly detectable in the cytosol and extracellular milieu, glycosylated proteins have been also identified in the nucleus including nucleoporins, transcription factors and hnRNPs (Hanover *et al.* 1987, Ferraro *et al.* 1991). Addition of saccharides to nuclear extracts of HeLa cells abolished splicing activity of Gal-1 and -3 suggesting carbohydrate dependent interaction (Dagher *et al.* 1995). Therefore, binding of Gal-12 to proteins of the spliceosome via the conserved CRD seems likely. However, it cannot be excluded that intracellular functions of galectins, although saccharide-inhibitable, are based on protein-protein interactions. It is possible that saccharide binding to the CRD induces a conformational change that disrupts the interaction of the galectin with other proteins. Intriguingly, similar to the observed subcellular distribution pattern of Gal-12, SR splicing factors undergo nuclearcytoplasmic shuttling in a cell-cycle dependent manner as well (Tripathi & Parnaik 2008). Translocation of SR proteins into the cytoplasm has been linked to RNA export where they stimulate translation (Huang *et al.* 2003). Binding of Gal-3 to mRNA especially during mitosis has been confirmed in the literature thereby contributing to enhanced half-life time of MUC4 mRNA known to be involved in proliferation (Coppin *et al.* 2017). Though direct interaction of Gal-12 with SR-proteins has not been demonstrated yet, it is reasonable to assume that this interaction may regulate its subcellular transport.

Galectins have attracted attention as potential modulators of tumor development and progression (Liu & Rabinovich 2005). Though little is known about the contribution of Gal-12 to tumorigenesis, our data provide the first evidence for the involvement of Gal-12 in alternative splicing and pre-mRNA processing, suggesting it as a potent modulator of cellular homeostasis of cells with a higher differentiation degree. Thus, our model cell line provides the basis for deciphering the biological consequence of re-expression of Gal-12 on CRC carcinogenesis at a molecular level in the future for the first time. In particular, identification of the galectin`s binding partners by Flag-tag mediated co-immunoprecipitation is now enabled.

Conflict of interest

The authors declare no conflict of interest.

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Author Contributions

JK, JG and EK designed the experiments. EK performed all experiments. JK, JG, EK and HS were involved in data analysis. HS helped with immunofluorescence staining experiments and was involved in critically revision of this article. JK, JG and EK wrote the manuscript. All authors read and approved the final version of this manuscript.

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