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Research paper

# Gelsolin interacts with LamR, hnRNP U, nestin, Arp3 and β-tubulin in human melanoma cells as revealed by immunoprecipitation and mass spectrometry

Antonina Joanna Mazur[a,*], Tomasz Radaszkiewicz[a,1], Aleksandra Makowiecka[a], Maria Malicka-Błaszkiewicz[a], Hans Georg Mannherz[b], Dorota Nowak[a]

[a] Department of Cell Pathology, Faculty of Biotechnology, University of Wroclaw, ul. Joliot-Curie 14a, 50-383 Wrocław, Poland
[b] Department of Anatomy and Molecular Embryology, Faculty of Medicine, Ruhr-University, Universitätsstr. 150, 44780 Bochum, Germany

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ABSTRACT

Gelsolin, a multifunctional actin binding protein, plays a not yet fully understood role in tumorigenesis. Therefore the goal of this study was to identify additional molecular partners of gelsolin in human melanoma cells, separately in the cytoplasmic compartment and cell nuclei. For this purpose we performed immunoprecipitation experiments based on a modified protocol followed by mass spectrometry. The obtained results were confirmed by Western blot analysis, proximity ligation assays and confocal microscopy. As expected gelsolin interacted with actin, in particular we demonstrate its interaction with cytoplasmic β and γ actins, and a newly discovered actin isoform, actbl2. As new gelsolin-interacting partners we identified the ribosomal protein Rpsa, also known as a non-integrin laminin receptor (LamR), and the heterogeneous nuclear ribonucleoprotein hnRNP U. Our data furthermore indicate that gelsolin interacts with particular components of the three cytoskeleton systems: nestin (intermediate filaments), Arp3 (actin cytoskeleton) and β-tubulin (microtubules). We also report for the first time that gelsolin is a constituent of midbodies, a tubulin containing structure formed at the end of cytokinesis.



## 1. Introduction

Gelsolin is a multifunctional actin binding protein and takes part in the dynamic reorganization of the actin cytoskeleton due to its capping, severing and under certain circumstances nucleating abilities (Mannherz et al., 2010; Nag et al., 2013). Gelsolin is a member of the gelsolin/villin family of actin binding proteins (AbP) and characterized by six homologous segments of about 14 kDa (Kwiatkowski et al., 1986). Gelsolin is present in the leading edge of lamellipodia or podosomes and invadopodia (Attanasio et al., 2011; Cervero et al., 2012; Mazur et al., 2010), i.e. structures responsible for the migration of macrophages and tumor cells. Gelsolin has been localized also in the cell nuclei of several tumor cell types, e.g. in astrocytoma (Ohnishi et al., 2009), in subpopulations of colorectal carcinoma cells (Zhuo et al., 2012) and in melanoma cell lines (our unpublished observations), but its possible nuclear function is still not fully elucidated. Among the members of the gelsolin family only supervillin possesses a nuclear localization sequence (NLS), whereas gelsolin has neither an NLS nor a nuclear export signal (NES) (Li et al., 2012). Published data show that intracellular gelsolin exists in several states of activation e.g. free gelsolin and $Ca^{2+}$-bound gelsolin, but only free gelsolin can translocate into the nucleus (Van den Abbeele et al., 2010). On the other hand, it was found that gelsolin can enter the cell nucleus in a complex with steroid hormone and its receptor (Ambrosino et al., 2010; Nishimura et al., 2003). Nishimura et al. (2003) postulated that formation of an androgen:androgen-receptor:gelsolin complex is crucial for the interaction of the activated receptor via nuclear actin with RNA polymerase II. Though these data suggest that steroid hormones may be responsible for the nuclear localization of gelsolin in melanoma cells, we postulate that gelsolin might have additional

*Abbreviations:* Arp3, actin-related protein 3; CF, cytosolic fraction; CK, commercial kit; Co-IP, co-immunoprecipitation/co-immunoprecipitate; F-actin, filamentous actin; G-actin, monomeric actin; *GSN*, gelsolin coding gene; HA, hemagglutinin tag; IgGs, immunoglobulins; hnRNP U, heterogeneous nuclear ribonucleoprotein U; IP, immunoprecipitation; LamR, laminin receptor; LC/MS, liquid chromatography/mass spectrometry; NF, nuclear fraction; PIC, Protease Inhibitor Cocktail; PLA, proximity ligation assay; Rpsa, ribosome associated protein; RT, room temperature; WB, Western blot; WCL, whole cell lysate.

* Corresponding author.
*E-mail address:* antonina.mazur@uwr.edu.pl (A.J. Mazur).
[1] Present address: Faculty of Science, Department of Experimental Biology, Masaryk University, Czech Republic.

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functions in the cell nucleus. Indeed, recent data have described an interaction of gelsolin with FBXO25, an F-box protein serving as a specificity factor for ubiquitin ligases found predominantly in FBXO25-associated nuclear domains (FAND) (Teixeira et al., 2010).

Recent reports have suggested that gelsolin exerts a role in the tumorigenesis either as a tumor suppressor or a tumor promoter. These contradictory hypotheses are based on observations of either a down-regulation of gelsolin (*GSN)* in e.g. breast, bladder, colon, gastric, kidney, lung, oral, ovarian, pancreatic and prostate cancer (as reviewed by Li et al. (2012)) or an up-regulation in non-small cell lung, urothelial, pancreatic and cervical cancers (Rao et al., 2002; Shieh et al., 2006; Thompson et al., 2007; Yang et al., 2004). Interestingly, a biphasic gelsolin expression was observed during the transition of oral precancerous lesions to oral cancers (Shieh et al., 2006). *GSN* down-regulation was noted in precancerous lesions, whereas *GSN* up-regulation was observed in oral cancer progression. Moreover, it was shown that gelsolin positively stimulates proliferation, migration and invasion of human oral squamous carcinoma cells (Deng et al., 2015).

We have shown previously that gelsolin is crucial for migration and invasion of colon adenocarcinoma and melanoma cell lines (Litwin et al., 2012, 2009). In colon adenocarcinoma cells overexpression of gelsolin resulted in a higher, whereas downregulation of *GSN* expression in melanoma cells resulted in a lowered migration potential. In both cases we observed dramatic changes in the organization of the actin cytoskeleton i.e. reduced polymerization state in gelsolin overexpressing cells and an increase in cytoplasmic stress fibers in the cells with silenced gelsolin expression reflecting the known F-actin severing activity of gelsolin. However, gelsolin was present in the cell nuclei of A375 cells even 72 h after triggering the downregulation of *GSN* expression, although the amount of gelsolin was clearly diminished in the cytoplasmic compartment (Litwin et al., 2012). These intriguing observations inspired us to study the nuclear gelsolin and its possible interaction partners in greater detail. For this study we selected established cell lines derived from human melanoma and aimed to identify molecular partners of gelsolin with special attention to possible interacting partners of nuclear gelsolin.

For this purpose we optimized the procedure to immunoprecipitate endogenous gelsolin from whole cell lysates (WCLs) and nuclear fractions (NFs) of melanoma cells. Since our aim was to focus on additional interactions of the endogenous gelsolin, we did not use cells transfected cells with overexpressed tagged gelsolin. Liquid chromatography/mass spectrometry (LC/MS) analysis of the co-immunoprecipitated complexes (Co-IPs) led to the identification of new protein partners interacting with endogenous gelsolin. The specificity of these partners was further confirmed by Western blotting (WB), proximity ligation assay (PLA) and microscopical colocalization analyses. We are confident that our data may have interesting implications for the biology of human melanoma and may trigger new studies on gelsolin's role in tumor cells.

## 2. Materials and methods

### 2.1. Antibodies and dyes

Rabbit anti-emerin (FL-254), mouse anti-GAPDH (H-12), goat anti-gelsolin (C-20), mouse anti-hnRPU U (3G6), rabbit anti-laminin-R (H-141), goat anti-laminin-R (F-18), mouse anti-nestin (10c2), rabbit anti-Arp3 (H-110), goat anti-HA (Y-11) and normal goat IgGs were obtained from Santa Cruz Biotechnology® Inc. (Heidelberg, Germany). Mouse anti-β actin (AC-15), mouse anti- γ actin (2-2.1.14.17), mouse anti-all isoactins (AC40), rabbit anti-all isoactins (C-11), mouse anti-tubulin β (TUB 2.1) and mouse anti-gelsolin (GS-2C4) antibodies were from Sigma–Aldrich® (Warsaw, Poland) and mouse anti-RNA polymerase II antibodies (8WG16) were from Covance (Poznan, Poland). Donkey anti-goat-Alexa Fluor®-488, donkey anti-mouse-Alexa Fluor®-568 and donkey anti-rabbit-Alexa Fluor®568 antibodies as well as Alexa Fluor®-568-labeled phalloidin and Alexa Fluor®-594-labeled DNase I were from Invitrogen™ (Warsaw, Poland).

### 2.2. Cell culture

A375, WM35, Hs294T, LS174T, MCF-7, HepG2 and A431 cells were from ATCC® (Lomianki, Poland). Cells were cultured according to suppliers' recommendations. WM9 cells were a kind gift of Prof. Andrzej Mackiewicz from Greater Poland Cancer Center in Poznan, Poland; this cell line can be bought from Coriell Institute (Camden, NJ, USA).

### 2.3. Steroids deprivation

A375 cells were rinsed three times with warm PBS and DCC DMEM medium containing 10% dextran charcoal (DCC)-stripped of FBS was added to the cells (Darbre et al., 1983). Medium deprived of steroids was changed every second day. After 7 days the cells were fixed and analyzed. DCC-FBS was prepared as follows. 50 ml of FBS were incubated with 1 g of dextran coated charcoal (Sigma–Aldrich®) for 45 min at 56 °C followed by an overnight incubation at 4 °C with gentle agitation. Next, serum was centrifuged at 1500 × *g* for 10 min and supernatant was filtrated through a 0.22 μm filter. DCC-FBS was aliquoted and frozen.

### 2.4. Synchronization of the cells

To synchronize the cells in mitotic phase we applied the Thymidine-Nocodazole Block (Jackman and O'Connor, 2001; Mullins and Mclntosh, 1982). Thymidine was added to a final concentration of 2 mM to a monolayer of cells at about 50% confluency in a ϕ 10 cm Petri dish. After 24 h the thymidine was removed by washing with sterile PBS. The cells were cultured for 3 h in fresh medium. Next nocodazole was added to a final concentration of 0.33 mM. After 12 h the nocodazole was removed by washing with sterile PBS. The Petri dishes with the cells submerged in fresh medium were knocked on a hard surface to dislodge the mitotic cells from the dish. These cells were centrifuged at 100 × *g* for 5 min at RT. Next the cells were plated on polylysine coated coverslips. 30 min later the cells were fixed with 4% formaldehyde (FA) for 20 min. Fixed cells were subjected either to immunostaining or to proximity ligation assays.

### 2.5. Western blot analysis

Cell lysates prepared by addition of the cytoskeletal-bound protein extraction buffer (10 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 20 mM $Na_4P_2O_7$, 2 mM $Na_3VO_4$, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate) were supplemented with protease inhibitor cocktail (PIC) and vortexed for 30 s. The lysates were three times frozen and thawed and subsequently centrifuged at 10,000 × *g* for 10 min at 4 °C; the supernatants were stored at −80 °C for further analysis. Protein concentration was estimated by the standard Bradford procedure (Bradford, 1976). Next, 10 or 30 μg of protein was subjected to SDS-PAGE using 10% gels (Laemmli, 1970), subsequently transferred to nitrocellulose (Towbin et al., 1979) and after blocking incubated with the primary antibodies overnight at 4 °C. After incubation with secondary antibodies conjugated to horseradish peroxidase (HRP) the immunoblots were developed using the Western blotting Luminol Reagent (Bio-Rad, Warsaw, Poland). Photos of blots were taken with ChemiDoc™ MP System (Bio-Rad), which were