

NUCLEAR LOCALIZATION OF BCR AND CORTACTIN INDICATES THEIR POTENTIAL ROLE IN REGULATION OF ACTIN BRANCHING IN NUCLEUS

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Aim: To study cellular localization of full-length breakpoint cluster region (BCR), Pleckstrin homology domain of BCR and cortactin and determine whether they can coexist in cell nucleus. **Materials and Methods:** HEK293T cell line was transfected with *pECFP-BCR*, *pEGFP-PH* and *pmTagRFP-N1-CTTN* using polyethyleneimine. Live cells were imaged in cell culture dishes with glass coverslip attached to the bottom with Leica SP8 STED 3D confocal microscope in the environmental chamber. Obtained images were processed and analyzed with Fiji software. **Results:** We identified colocalization of full-length BCR and cortactin in nucleus of cell undergoing terminal phase of cell division. We did not observe nuclear localization of cortactin in non-dividing cell. Both Pleckstrin homology domain and full-length BCR exhibited cytoplasmic as well as nuclear localization. **Conclusions:** Colocalization of BCR with cortactin in cell nucleus indicates their potential role in regulation of actin network allowing for the maintenance of nuclear architecture and DNA integrity.

Key Words: chronic myeloid leukemia, BCR-ABL, cortactin, actin branching, nuclear integrity.

DOI: 10.32471/exp-oncology.2312-8852.vol-43-no-1.15811

Chronic myeloid leukemia is caused by the translocation between 9 and 22 chromosome and generation of BCR-ABL p210 fusion oncoprotein. Understanding molecular mechanisms involving different phenotype of BCR-ABL-positive myeloproliferative disorders is crucial for differential diagnosis and effective treatment. In previous work we identified 23 potential interaction partners of Pleckstrin homology (PH) domain of breakpoint cluster region (BCR) by mass-spectrometry [1]. Cortactin was among these possible binding partners. Main function of cortactin is modulating actin branching in tandem with actin-related proteins 2/3 (ARP 2/3) complex [2]. Cortactin has multiple functions and affects tumor invasiveness [3], cell adhesion and motility [4, 5], and actin scaffolding during fission of clathrin-coated vesicle [6–9]. While ABL mainly localizes in cell nucleus, BCR and BCR-ABL have cytoplasmic localization despite BCR having nuclear localization signal (NLS) [10]. Some data indicate that BCR-ABL can shuffle from cytoplasm to nucleus in response to genotoxic stress [11]. Other study demonstrated that explicit targeting of BCR-ABL to nucleus via four NLS caused apoptosis [12]. Due to the increased level of nuclear c-ABL, the level of F-actin in the nucleus is augmented. Tyrosine kinase activity of ABL does not affect overall level of F-actin in the nucleus, but is required for actin bundle formation [13]. It is known that phosphorylation of cortactin regulates its ability to branch actin [14–18]. While cortactin is mostly cytoplasmic protein, there is some evidence that it can move to the nucleus after acetylation in response to stress [19]. In this study, we attempted to determine whether PH domain and full length BCR

are able to localize in the cell nucleus of living cells. These data may reveal potential role of BCR and cortactin in actin remodeling supporting such crucial functions as maintaining chromatin organization and response to DNA damage.

MATERIALS AND METHODS

Cell culture. HEK293T cells were obtained from mammalian cell culture repository of EMBL, Heidelberg. HEK293T cells were grown in DMEM supplied with 10% fetal bovine serum (Biowest, South Korea) and 50 µg/ml penicillin-streptomycin antibiotic mix at 37 °C, 5% CO₂ and 100% relative humidity.

Plasmids and vectors. *ECFP-Bcr* vector encoding full-length BCR tagged with enhanced cyan fluorescent protein was a gift from John Groffen & Nora Heisterkamp (Addgene plasmid № 36415; <http://n2t.net/addgene:36415>; *RRID:Addgene_36415*) [20]. Mammalian expression vector *pEGFP-PH* encoding PH domain of BCR tagged to enhanced green fluorescent protein was created and described previously [1]. Cortactin sequence was amplified with CTTN-F (ATGTGGAAAGCTTCAGCAGG) and CTTN-R (AGCTCCACATAGTTGGCTGG) primers. Derived DNA fragment was purified from agarose gel on silica columns and ligated to *pBluescriptSKII+* plasmid on EcoRV site. It was further subcloned into *pmTagRFP-N1* (Michael Davidson, Florida State University) vector on BamHI and Sall sites to obtain cortactin sequence tagged with red fluorescent protein that can be expressed in mammalian cells. Plasmids were isolated from transformed overnight culture of *E. coli* Mach1 cells grown on selective antibiotic by alkaline lysis method [21]. Purification from RNA was done by selective CaCl₂ precipitation [22].

Fluorescence microscopy and image analysis.

Images were obtained on Leica SP8 STED 3D microscope with 100x oil objective and 1.4 numerical aperture. For excitation of ECFP, white light laser was set to 456 nm wave-

Submitted: November 25, 2020.

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Abbreviations used: ARPs – actin-related proteins; BCR – breakpoint cluster region; NLS – nuclear localization signal; PEI – polyethyleneimine; PH – Pleckstrin homology.

length; for excitation of mTagRFP white light laser was set to 564 nm wavelength, for excitation of EGFP — 488 nm laser wavelength was used. Laser intensity was set adjusted to obtain good signal-to-noise ratio and avoid photodamage of cells. Effective pixel size was calculated according to Nyquist criteria. Before transfection, cells were grown to 50–70% confluency, 1 µg of purified plasmid was mixed with triple volume of 1 µg/µl polyethylenimine (PEI) in 200 µl of DMEM without FBS and incubated 20 min at room temperature. Following incubation, DNA/PEI was added dropwise to cell culture plate [23]. Transfected cells were imaged 12–24 h after transfection. Cell culture plates with glass microslides attached to the bottom were used for live cell imaging. Cells were imaged in environmental chamber attached to the microscope that provides temperature, humidity and CO₂ control to maintain 37 °C, 5% CO₂ and 100% relative humidity. DMEM without phenol red and with 20 mM HEPES was used in live cell imaging to avoid autofluorescence.

Fiji software was used for image processing and analysis. Gaussian blur filter of 1 pixel radius was applied to raw images. Afterwards, they were deconvolved in Deconvolution Lab 2 plugin using 10 iterations of Richardson-Lucy total variation algorithm. Final images were arranged using EzFig plugin. Overlap analysis between two proteins of interest was done in JaCOP plugin using Manders coefficients [24, 25].

RESULTS AND DISCUSSION

We have identified that both PH domain and full length BCR localized in nucleus and cytoplasm. Cortactin has predominantly cytoplasmic localization in non-dividing cells. However, we were able to detect cells on the terminal phase of nuclei separation where there are clear signs of cortactin presence in the nuclear structures (Figure). In dividing cell, BCR is more concentrated in cell nucleus, while in non-dividing cell it has more uniform distribution between nucleus and cytoplasm. Interestingly, in these cortactin localization spots, cortactin was surrounded by the rings of BCR protein. In non-dividing cells, cortactin was exclusively cytoplasmic, while PH domain of BCR has both nuclear and cytoplasmic localization (Table).

Cortactin regulates actin branching, and possibly this activity of cortactin is effective in different cellular locations. The data that some types of ARPs are nuclear are in favor of this assumption [26]. Possibly, cortactin may interact with nuclear ARPs to promote actin branching. Actin branching was observed after NLS-cAbl is expressed in cell nucleus [13]. Because tyrosine kinase activity of Abl was essential for the formation of actin bundles, one may suggest that activation of cortactin through tyrosine phosphorylation may promote this activity [27]. Phosphorylated form of cortactin was found associated with centrosomes [28]. Apart from controlling cell division and mitotic spindle formation centrosomes also act as actin organizing center [29]. Taken together, this may be an indirect indication that actin branching function of cortactin may be modulated by tyrosine kinase activity of c-Abl. Our previous bioinformatic analysis demon-

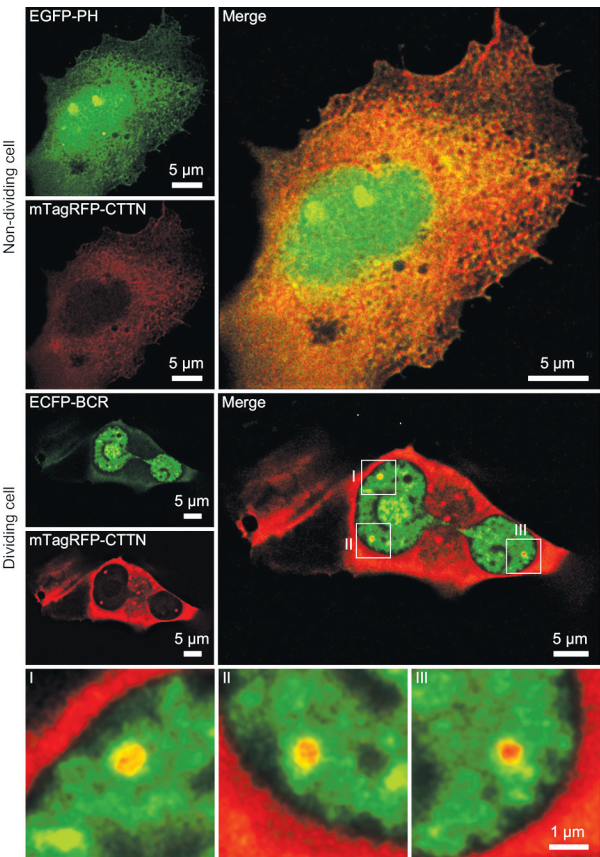


Figure. Live cell confocal microscopy of HEK293T cells transfected with ECFP-BCR + mTagRFP-CTTN and EGFP-PH + mTagRFP-N1-CTTN. Image illustrates difference in subcellular localization of cortactin with full-length BCR and PH domain of BCR in non-dividing cells and cells on terminal stage of division that are characterized by the presence of contractile ring. Subfigures I–III illustrate formation of nuclear structures between cortactin and BCR, where several point-like locations of cortactin are surrounded by BCR

Table. Manders overlap coefficients between cortactin and full-length BCR and PH domain of BCR in dividing and non-dividing HEK293T cells

Protein pair	Manders M1 +/- SE (fraction of first protein overlapping second protein)	Manders M2 +/- SE (fraction of second protein overlapping first protein)
BCR/cortactin (dividing cells)	0.032 ± 0.007	0.057 ± 0.010
PH/cortactin (non-dividing cells)	0.297 ± 0.030	0.815 ± 0.057

Note: Statistical significance of difference between Manders coefficients of dividing and non-dividing cells was confirmed by *t*-test (*p* < 0.05).

strated that both Src and c-Abl kinases can phosphorylate the same tyrosine residues in cortactin [30]. The recent studies unravel that actin is a part of chromatin remodeling complexes, take part in transcription, ribonucleo-protein binding, DNA repair and chromatin organization in the nucleus [31–32]. These activities require highly coordinated action with actin-binding proteins, to which cortactin and c-Abl belong. Little is known about the role of BCR in actin binding. BCR coil-coiled oligomerization domain is required for BCR-ABL transforming function and promotes F-actin binding of Abl [33]. We have recently discovered that BCR alone colocalizes with cortactin and points of actin branching (unpublished data). Therefore, BCR can also affect actin branching even without c-Abl fusion partner. PH domain of BCR exhibits lipid binding

function and promotes membrane invagination during cytoskeleton remodeling [34–36]. Similar organizing functions of PH domain may be essential to maintain nuclear functions of actin and cortactin. Additional evidence of the role of PH domain in these processes is that it may interact with SMC1 protein [1], that is responsible for structural maintenance of chromosomes [37–38]. To summarize, our data on colocalization between cortactin and full-length BCR in cell nucleus may be an indication that they are important for structural and functional maintenance of nuclear function during cell division and in response to stress.

These data may provide understanding of possible intervention strategies in order to treat BCR-ABL-positive myeloproliferative disorders. Future studies will reveal whether complexes between BCR, actin and cortactin exist in cell nucleus of dividing and non-dividing cells and how cortactin phosphorylation affects formation of these complexes.

FINANCIAL SUPPORT

This work was supported by the I-Next grant “Spatial distribution of BCR-ABL, cortactin, clathrin and β -tubulin on subdiffraction level and their role in clathrin-mediated endocytosis” (project id: 8230) under the framework of Horizon 2020 program.

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**ЯДЕРНА ЛОКАЛІЗАЦІЯ BCR І КОРТАКТИНУ
ВКАЗУЄ НА ЇХ ПОТЕНЦІЙНУ РОЛЬ В РЕГУЛЯЦІЇ
РОЗГАЛУЖЕННЯ АКТИНУ В ЯДРІ**

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Мета: Дослідити клітинну локалізацію повнорозмірної ділянки кластерної точки розриву (breakpoint cluster region — BCR), PH-домена BCR і кортактину та визначити, чи можуть вони співіснувати в ядрі клітини. **Матеріали та методи:** Клітинна лінія HEK293T була трансфорована *pECFP-BCR*, *pEGFP-PH* та *pmtagRFP-N1-CTTN* з використанням поліетиленіміну. Живі клітини візуалізували в чашках для культивування клітин зі скляним покривним скельцем, прикріпленим до дна, за допомогою конфокального мікроскопа Leica SP8 STED 3D у спеціальній інкубаційній камері. Отримані зображення були оброблені та проаналізовані за допомогою програмного забезпечення Fiji. **Результати:** Ми ідентифікували колокалізацію повнорозмірного BCR і кортактину в ядрі клітин, що проходили термінальну фазу клітинного поділу. Ми не спостерігали ядерної локалізації кортактину в клітинах, які не ділилися. І PH-домен, і повнорозмірний BCR локалізувалися як в ядрі, так і в цитоплазмі. **Ви-**