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PH domain of BCR provides colocalization of full-length BCR with centrosome together with cortactin to facilitate actin-organizing function

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Chromosomal translocation between 9 and 22 chromosomes leads to the fusion of *bcr* and *abl* genes. Because of different breakpoints in *bcr* gene three forms of chimeric BCR-ABL proteins exist – p230, p210, and p190. BCR-ABL^{p190} lacks Pleckstrin homology (PH) domain of BCR and is associated with acute lymphoblastic leukaemia. In contrast, BCR-ABL^{p210} has PH domain and occurs during chronic myeloblastic leukaemia. BCR-ABL can bind to centrosomes, which function as a regulating center of cell division and spindle formation during mitosis. Cortactin, the main function of which is actin branching, was previously identified by mass-spectrometry as one of the potential partners interacting with the PH domain of BCR. **Aim.** To determine whether BCR and cortactin colocalize with centrosomes and to study a possible role of PH domain in such colocalization. **Methods.** Mammalian cell culturing, immunofluorescence analysis, fluorescence microscopy of live cells. **Results.** In the present work we show that both full-length BCR protein and PH domain colocalize with centrosome together with cortactin in live HEK293T cells. We also demonstrate that BCR colocalizes with γ-tubulin and the points of actin branching in fixed K562 cells. Using anti-ABL and anti-BCR antibodies we also show that colocalization with the actin branching center is typical for BCR-ABL and BCR, but not for ABL alone. **Conclusions.** PH domain of BCR is required for colocalization of BCR or BCR-ABL with centrosome. Together with cortactin, BCR-ABL can affect centrosome function through deregulation of actin branching or abnormal phosphorylation, which can be a matter of further research.

K e y w o r d s: BCR-ABL, centrosome, cortactin, CML.

Introduction

The chromosomal translocation between 9 and 22 chromosomes leads to generation of Philadelphia chromosome [1] and promotes the development of different types of myelo-

proliferative disorders. The phenotype of myeloproliferative disorder is usually determined by the type of chimeric protein generated as a result of translocation and fusion of *bcr* and *abl* genes. Acute lymphoblastic leukaemia is associated with BCR-ABL^{p190} protein, chronic myelogenous leukaemia − with BCR-ABL^{p210} protein, chronic neutrophilic leukaemia – with BCR-ABL^{p230} protein. These three proteins differ only in the presence or absence of certain domains of BCR, whereas ABL part remains constant in all three types. We previously identified that PH domain of BCR potentially interacts with 23 proteins of K562 cell line, which was originally derived from CML patient [2]. We focused on the cortactin as a promising candidate for interaction with PH domain because cortactin is frequently involved in cancer progression. Primarily its role in cancer progression is caused by abnormalities in actin regulation leading to increased invasiveness and altered adhesion [3]. This, however, is more common for solid tumors with different subpopulations of cancer cells [4–8]. The role of CTTN in progression of blood cancer, which is a result of clonal expansion of the most proliferatively active cells, is still poorly understood. In one of our previous work we discovered that CTTN and PH domain of BCR are colocalized near the cell nucleus [9]. However, we could not identify the structure they were attached to. It is known that centrosome is a microtubule-organizing center that is located near the cell nucleus and regulate the important processes such as the formation of mitotic spindle during cell division [10]. Moreover, it is known that centrosomes are actin-organizing unit as well [11]. The previous studies showed that leukemic cells disrupted the centrosome function and that the BCR-ABL protein was among the centrosome-associated proteins as it was demonstrated by co-immunoprecipitation [12]. Another study indicates that [the] phosphorylated form of CTTN is associated with centrosome [13]. In this study we aim to determine whether the full-length BCR and PH domain of BCR colocalize with centrosomes as well as whether CTTN is involved in this process.

Materials and Methods

Plasmid vectors and genetic constructs. Vector EGFP-PH was created previously in our lab [2]. CTTN sequence was amplified using pOTB7-CTTN vector with CTTN-F (5'ATGTGGAAAGCTTCAGCAGG) and CTTN-R (5'AGCTCCACATAGTTGGCTGG) primers and cloned into pBluescriptSKII(+) vector on EcoRV site. Further, CDS of cortactin nucleotide sequence was subcloned into pmTagRFP-N1 (Michael Davidson, Florida State University) on BamHI and SalI sites. Plasmids were isolated from the transformed overnight culture of *E.coli* NEB Turbo cells grown on selective antibiotic by the alkaline lysis method [14]. To obtain transfection-grade plasmid DNA, purification was done by CaCl2 precipitation followed by NaCl/PEG6000 precipitation [15].

Cultivation of HEK293T and K562 cells. HEK293T and K562 cells were obtained from cell line bank of EMBL, Heidelberg. HEK293T cells were grown in DMEM media with L-glutamine containing 10 % fetal bovine serum, 100 µg/ml streptomycin, 10000 U penicillin at +37 °C, 5 % CO_2 and 95 % relative humidity. K562 cells were grown in RPMI-1640 media with L-glutamine containing 10 %

fetal bovine serum, 100 µg/ml streptomycin, 10000 U penicillin at +37 °C, 5 % CO_2 and 95 % relative humidity. The cell lines were tested negative for mycoplasma before performing experiments.

Antibodies and dyes. Rabbit primary polyclonal anti-BCR antibody (Abclonal, AA0068), mouse primary monoclonal anti-γ-tubulin antibody (Thermo Fisher Scientific, MA1–19421), mouse primary anti-ABL antibody (Calbiochem, OP20), anti-mouse Alexa-555 conjugated secondary antibodies (Abclonal, AS057), anti-rabbit secondary Alexa-647 conjugated secondary antibodies (Abclonal, AS060), FITC-phalloidin (Sigma-Aldrich, P5282), DAPI (Sigma-Aldrich, D9542), SiR-tubulin (Spirochrome, SC002).

Fixation and Staining. Before fixation, growth media was carefully aspirated from cells. After that the cells were fixed in 4 % paraformaldehyde (Sigma-Aldrich, 158127) in PBS for 15 min at room temperature. Following fixation, the cells were washed thrice with PBS and permeabilized in 0.1 % Triton X-100 (Serva) in PBS for 10 minutes at room temperature. Next, cells were washed thrice with PBS, and blocked in 1 % Bovine Serum Albumine (Sigma-Aldrich, 05470) in PBS for 1 hour at room temperature. Blocking solution was discarded and corresponding solution of primary antibodies was added to the cells and incubated overnight at +4 °C. Following incubation, cells were washed thrice with PBS and corresponding solution of secondary antibodies was added and incubated for 1 hour at room temperature. After incubation with secondary antibodies the cells were washed thrice with PBS and mounted on microslides with Mowiol 4–88 (Sigma-Aldrich, 81381) hardening me-

dium [16] containing 2.5 % DABCO (Sigma-Aldrich, D27802) as antifade reagent. Cells were ready for imaging 24 hours after mounting. For fixation and staining of K562 cells, the modified protocol was used that involves centrifugation for discarding the supernatant.

Fluorescence Microscopy. For fixed and live cell imaging we have used Carl Zeiss LSM 510 Meta confocal microscope with 100x oil objective and 1.4 numerical aperture. For excitation of ECFP we used 405 nm laser line, for excitation of EGFP 488 nm laser line was used, for excitation of mTagRFP and SiR-tubulin/Alexa-647–532 nm and 633 nm laser lines, respectively. The line-by-line sequential acquisition mode was used for live imaging. The laser intensity and detection channels were adjusted to avoid cross-excitation and spectral bleedthrough. Effective pixel and voxel sizes were calculated according to Nyquist criteria [17, 18]. Live cell imaging was done at +37 °C and 5 % CO2 in custom designed CNC machined anodized aluminum slides with pads designed to fit 18*18 mm glass coverslips. Briefly, slides are of size of standard glass microslides and contain rectangular hole with pads. Pads are covered with coverslips, the growth medium is placed between two slides. Coverslips were sealed using food-grade silicon grease.

Image processing and analysis. Image analysis and processing were done in Fiji software [19]. Colocalization analysis was performed in JaCOP plugin [20] using Manders coefficients [21]. Before analysis, the Gaussian deblur filter with radius of 1 pixel was applied to the image. Background was measured by line profiler and subtracted from the images if necessary. The images were then deconvolved

with Deconvolution Lab 2 plugin [22] using Richardson-Lucy Total Variation algorithm [23] with theoretical PSF generated by PSF Generator plugin [24]. The number of iterations for deconvolution ranged from 5 to 20 and was chosen empirically for the best structure preservation and absence of artifacts. Generation of final montage of image sets was done using Fiji EzFig plugin [25].

Results and Discussion

Disruption of centrosome function often leads to the development of different types of cancer [26]. These abnormalities may be caused by alteration in mitotic spindle formation and consequent deregulation of cell cycle and division [27]. Centrosome abberations are the well known feature of BCR-ABL-positive chronic myelogenous leukaemia [28]. It is still undetermined, whether it was a direct action of chimeric protein or cascade of regulatory events indirectly initiated by it. Moreover, centrosome also plays a role as actin organizing center, which promotes actin nucleation by WASH and Arp2/3 [11]. Cortactin in its phosphorylated form is able to bind to centrosome [13]. The cortactin-mediated actin branching typically involves Arp2/3 complex and dynamin [29–32]. Dynamin binds to membrane by its PH domain and provides coordinated cytoskeleton-membrane rearrangements [33]. We suggest that PH domain of BCR may have the similar function because it possesses strong affinity to phoshotidyl-inositol-phosphates [2]. In centrosomes cortactin mediates actin scaffold for separation of centrosomes during mitosis [13]. Because it is one of the potential interaction partner of PH domain of BCR it is interesting to determine whether this protein is able to colocalize with centrosome together with BCR-ABL. Here we show that indeed cortactin colocalizes with centrosomes both with PH domain of BCR and with full-length BCR in live HEK293T cells. To calculate the percentage of cooccurrence between two proteins we have taken Manders coefficient equal to 1 as 100 % colocalization. In HEK293T live cells 40.5 % of tubulin overlapped CTTN and 32.6 % of CTTN overlapped tubulin for colocalization analysis between SiR-Tubulin and mTagRFP-N1-CTTN, 63.7 % of tubulin overlapped BCR and 27.6 % of BCR overlapped tubulin for colocalization analysis between SiR-Tubulin and ECFP-BCR, 87.8 % of CTTN overlapped BCR and 62 % of BCR overlapped CTTN for mTagRFP-N1-CTTN and ECFP-BCR (Fig. 1, n=3). Colocalization analysis of EGFP-PH and mTagRFP-N1-CTTN showed that 34.2 % of PH domain of BCR was also found to colocalize with cortactin and 62.6 % of cortactin overlapped PH domain, it was also found that cortactin and PH domain overlapped centrosomes resulting in 12.9 % of PH domain overlapping tubulin and 54.1 % of cortactin overlapping tubulin, tubulin overlapped PH domain and cortactin in fraction of 35.4 % and 60.1 % respectively (Fig. 2, n=3). SiR-tubulin is a stain for live cell imaging of tubulin that can detect both filamentous tubulin and centrosomal region. Filamentous tubulin consists of α- and β-tubulin subunits [34] whereas centrosome consists mainly of γ-tubulin [35, 36]. We have used anti-γ-tubulin antibodies to check whether BCR is able to colocalize with centrosomes in fixed K562 cells. We detected that 65.6 % of BCR overlapped γ-tubulin and 49.6 % of γ-tubulin overlapped BCR. We also found
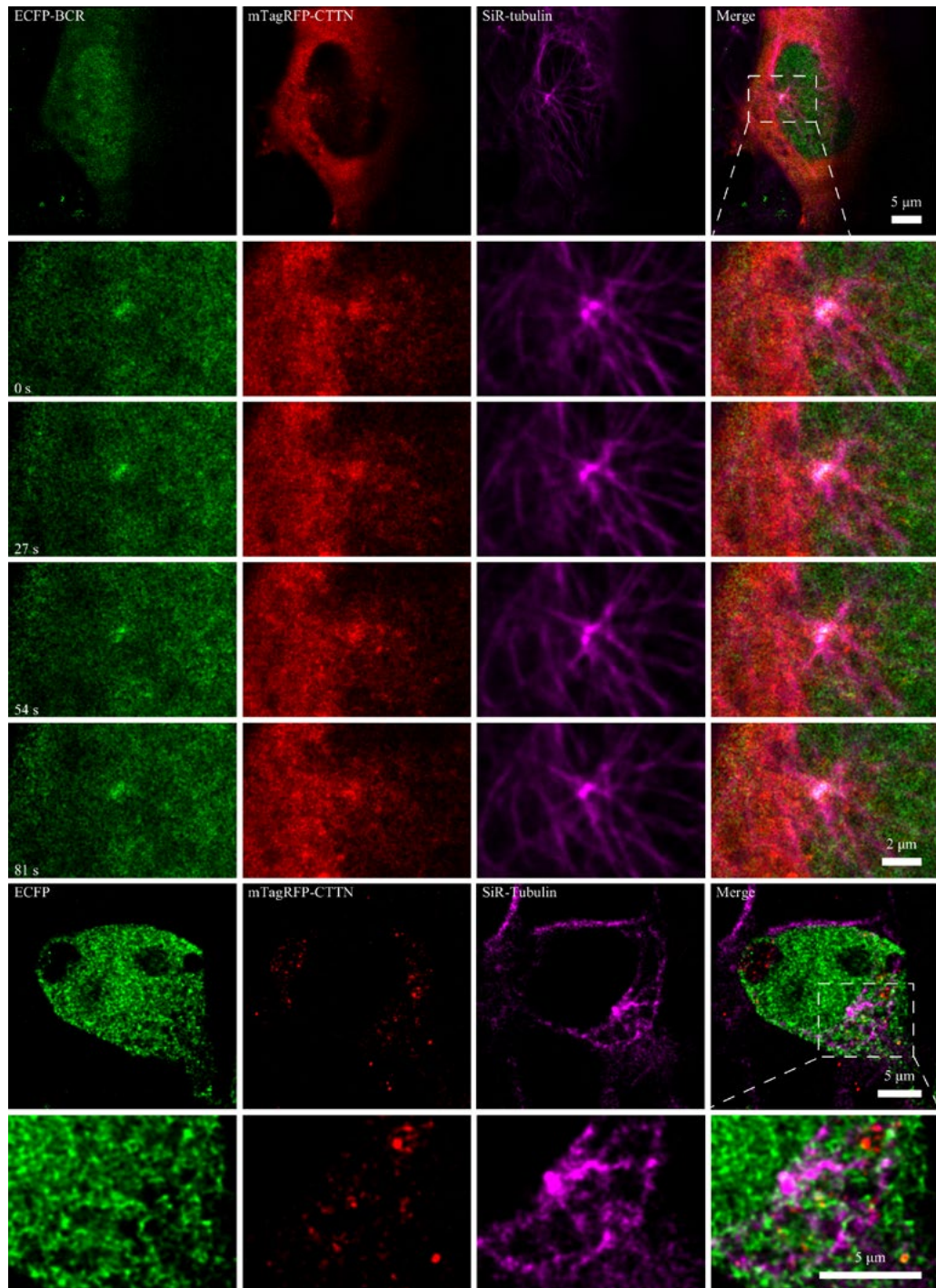
Fig. 1. Live cell imaging of HEK293T cells transfected with pECFP-BCR, ECFP, pmTagRFP-N1-CTTN vectors and stained with SiR-Tubulin.
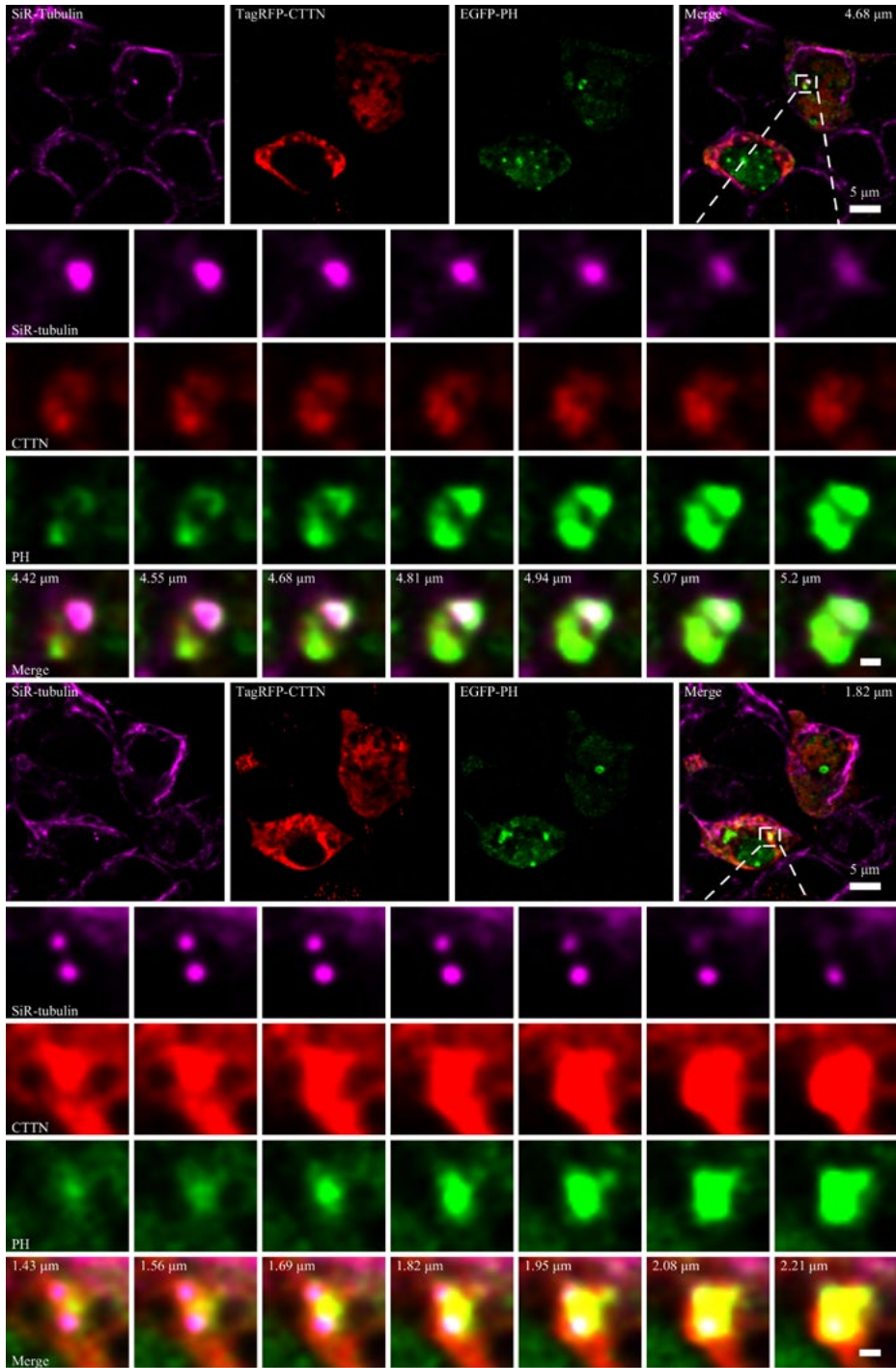
Fig. 2. Live cell confocal image of two projections of HEK293T cells transfected with EGFP-PH, pmTagRFP-N1-CTTN and stained with SiR-Tubulin. Scale bar on enlarged regions of interest indicates 500 nm.

that 83.2 % of BCR and 73.8 % of γ-tubulin overlapped actin, 36.6 % of actin overlapped BCR and 38 % of actin overlapped γ-tubulin (Fig. 3, n=3). Colocalization between BCR and γ-tubulin occurred in the points of actin branching. Such colocalization indicates a potential role of BCR-ABL in actin branching center. We may suggest that overlapping of BCR-ABL with cortactin in the centrosomal region may initiate actin branching not synchronized with the cell division cycle. It is also possible that other centrosomal proteins may be affected by the constant tyrosine-kinase activity of BCR-ABL. We also detected that the fusion protein BCR/ABL appears at the points of actin branching by staining fixed K562 cells with anti-BCR and anti-ABL antibodies and FITC-phalloidin. We have found that 75.6 % of BCR overlapped ABL and 67.7 % of ABL overlapped BCR, 87.6 % of

Table 1. **Overlap analysis between different pairs of proteins using Manders coefficents.**

Protein pair	Manders M1	Manders M2
EGFP-PH/SiR-tubulin	0.129	0.354
EGFP-PH/mTagRFP-CTTN	0.342	0.626
mTagRFP-CTTN/SiR-tubulin (Fig. 2)	0.541	0.601
ECFP-BCR/mTagRFP-CTTN	0.62	0.878
ECFP-BCR/SiR-tubulin	0.276	0.637
mTagRFP-CTTN/SiR-tubulin (Fig. 1)	0.326	0.405
anti-BCR/anti-γ-tubulin	0.656	0.496
anti-BCR/actin (FITC-phalloidin)	0.832	0.366
Actin (FITC-phalloidin)/anti-γ-tubulin	0.38	0.738
anti-BCR/anti-ABL	0.759	0.677
anti-BCR/actin (FITC-phalloidin)	0.876	0.245
anti-ABL/actin (FITC-phalloidin)	0.893	0.307
ECFP/mTagRFP-CTTN	0.008	0.292
ECFP/SiR-tubulin	0.115	0.162
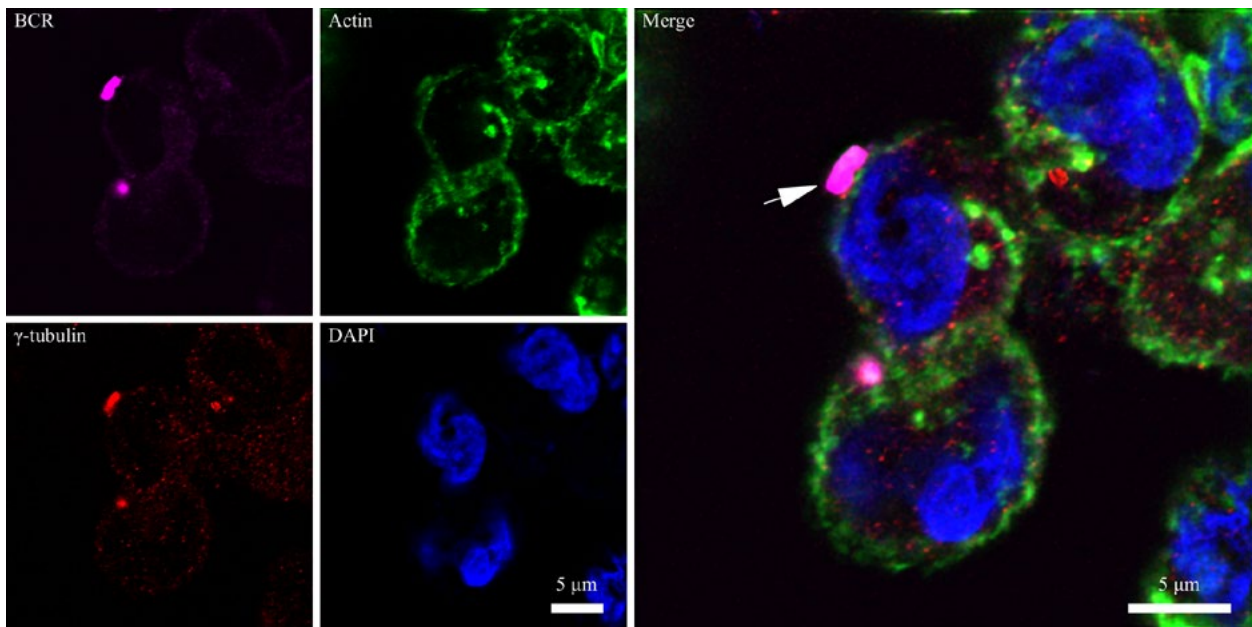
Fig. 3. Confocal image of fixed K562 cells stained with anti-BCR, anti-γ-tubulin, phalloidin-FITC and DAPI. Negative controls depict secondary antibodies without corresponding target primary antibodies.

BCR and 89.3 % of ABL overlapped with actin, 24.5 % actin overlapped with BCR and 30.7 % of actin overlapped with ABL (Fig. 4, n=5). Overlap analysis between different proteins and empty ECFP as the control sample (Fig. 1, n=3) is summarized in Table 1.
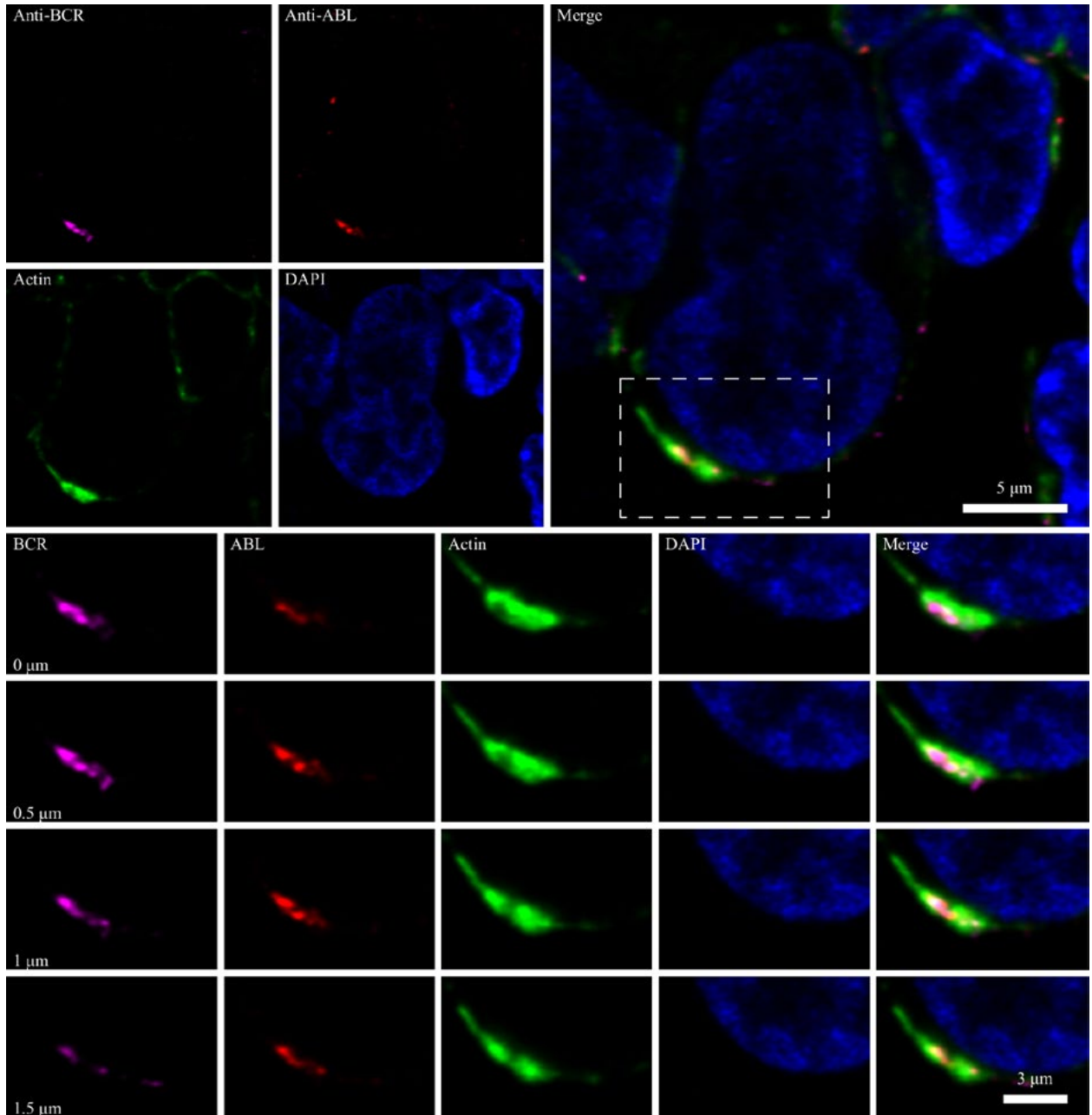
Fig. 4. Confocal image of fixed K562 cells stained with FITC-phalloidin, anti-BCR and anti-ABL antibodies, and DAPI.

Interestingly, ABL protein alone does not colocalize with centrosome without BCR, as we may see using anti-ABL and anti-BCR antibodies, despite the fact that it has actin-binding domain. Our results show that cortactin and BCR-ABL may be involved in centrosomal abberations possibly by affecting its actin-organizing function. Further study should include creating deletion mutants of BCR that lack PH domain and determining whether this will affect colocalization with centrosome and cortactin. The results may identify cortactin as one of the potential therapeutic targets for treatment of CML.

Funding

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Домен PH білка BCR забезпечує колокалізацію повнорозмірного BCR з центросомою разом з кортактином для забезпечення актиноорганізуючої функції.

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Хромосомна транслокація між 9 і 22 хромосомами призводить до злиття генів *bcr* та *abl*. Через різні точки розриву гена *bcr* існують три форми химерних білків BCR-ABL - p230, p210 та p190. BCR-ABL^{p190} не має домену гомології Плекстрину (PH) BCR і асоціюється з гострим лімфобластним лейкозом. З іншого

боку, BCR-ABL^{p210} має домен PH і виникає під час хронічного мієлобластного лейкозу. BCR-ABL може зв'язуватися з центросомами, які функціонують як регулюючий центр поділу клітини та формування веретена поділу під час мітозу. Кортактин, основною функцією якого є розгалуження актину, раніше був визначений за допомогою мас-спектрометрії одним із потенційних партнерів взаємодії домену PH BCR. **Мета.** Визначити, чи колокалізуються BCR та кортактин з центросомами та можлива роль домену PH у такій колокалізації. **Методи.** Культивування клітин ссавців, імунофлуоресцентний аналіз, флуоресцентна мікроскопія живих клітин. **Результати.** У цій роботі ми показуємо, що як повнорозмірний білок BCR, так і домен PH можуть колокалізуватися з центросомою разом з кортактином у живих клітинах HEK293T. Ми також показали, що BCR колокалізується з γ-тубуліном та точками розгалуження актину у фіксованих клітинах K562. Використовуючи анти-ABL та анти-BCR антитіла, ми також показуємо, що колокалізація з центром розгалуження актину є типовою для BCR-ABL та BCR, але не для виключно ABL. **Висновки.** Домен PH BCR необхідний для колокалізації BCR або BCR-ABL з центросомою. Разом з кортактином BCR-ABL може впливати на функцію центросоми через дерегуляцію розгалуження актину або аномальне фосфорилювання, що може бути предметом подальших досліджень.

Ключові слова: BCR-ABL, центросома, кортактин, ХМЛ.

Домен PH BCR обеспечивает колокализацию полноразмерного BCR с центросомой вместе с кортактином для обеспечения функции организации актина

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Хромосомная транслокация между 9 и 22 хромосомами приводит к слиянию генов *bcr* и *abl*. Из-за разных точек разрыва в гене bcr существуют три формы химерных белков BCR-ABL - p230, p210 и p190. BCR-ABL^{p190} не имеет домена гомологии плекстрина (PH) BCR и связан с острым лимфобластным лейкозом. Напротив, BCR-ABL^{p210} имеет домен PH и возникает при хроническом миелобластном лейкозе. BCR-ABL может связываться с центросомами, которые действуют как регулирующий центр клеточного деления и образования веретена деления во время митоза. Кортактин, основной функцией которого является разветвление актина, ранее был идентифицирован масс-спектрометрией как один из потенциальных партнеров взаимодействия с PH доменом BCR. **Цель.** Определить, колокализуются ли BCR и кортактин с центросомами, и возможную роль домена PH в такой колокализации. **Методы.** Культивирование клеток млекопитающих, иммунофлуоресцентный анализ, флуоресцентная микроскопия живых клеток. **Результаты.** В настоящей работе мы показываем, что как полноразмерный белок BCR, так и домен PH могут колокализироваться с центросомой вместе с кортактином в живых клетках HEK293T. Мы также продемонстрировали, что BCR колокализуется с γ-тубулином и точками ветвления актина в фиксированных клетках K562. Используя антитела против ABL и против BCR, мы также показываем, что совместная локализация с центром ветвления актина типична для BCR-ABL и BCR, но не для одного лишь ABL. **Выводы.** Домен PH BCR необходим для колокализации BCR или BCR-ABL с центросомой. Вместе с кортактином BCR-ABL может влиять на функцию центросом за счет нарушения регуляции ветвления актина или аномального фосфорилирования, что может быть предметом дальнейших исследований.

Ключевые слова: BCR-ABL, центросома, кортактин, ХМЛ.