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# Identifying the cellular location of brain cytoplasmic 200 RNA using an RNA-recognizing antibody

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**ABSTRACT**

Brain cytoplasmic 200 RNA (BC200 RNA) is a neuron-specific non-coding RNA that has been implicated in the inhibition of local synaptodendritic protein synthesis. It is also highly expressed in some cancer cells. Although BC200 RNA has been shown to inhibit translation in vitro, the cellular location of this inhibition was previously unknown. In this study, we used a BC200 RNA-recognizing antibody to identify the cellular locations of BC200 RNA in HeLa cervical carcinoma cells. We observed punctate signals in both the cytoplasm and nucleus, and further found that BC200 RNA colocalized with the p-body decapping enzyme, DCP1A, and heterogeneous nuclear ribonucleoprotein E2 (hnRNP E2). The latter is a known BC200 RNA-binding partner protein and a constituent of p-bodies. This suggests that BC200 RNA is localized to p-bodies via hnRNP E2.

## INTRODUCTION

In 1993, brain cytoplasmic 200 RNA (BC200 RNA) was first identified by Tiedge's group as a non-coding RNA expressed in human neuronal cells (1). A possible correlation between BC200 RNA and cancer was reported by the same group in 1997 (2). The authors detected BC200 RNA in human germ cells and several immortalized cell lines that did not originate from neurons. Moreover, their in situ hybridization experiments showed that BC200 RNA was abnormally overexpressed in various cancers, including those of the breast, lung, tongue, esophagus, ovary, and cervix (2). A later study found that BC200 RNA was expressed particularly high levels in invasive breast cancer cells (3). These findings suggest that BC200 RNA can contribute to tumorigenesis. Although there is some controversy (4), recent studies have suggested that BC200 RNA helps cancer cells avoid apoptosis and may enhance metastasis (5,6). For example, BC200 RNA was found to upregulate Bcl-xL, an isoform of Bcl-x, in breast cancer cells by altering its splicing pattern; this upregulation of Bcl-xL enabled cells to avoid apoptosis (5). Moreover, in non-small-cell lung cancers, BC200 RNA was shown to be induced by the transcription factor, c-Myc, which is highly associated with cancer metastasis, and the induced RNA was found to enhance cellular motility, thereby possibly promoting metastasis (6).

BC200 RNA is known to inhibit the initiation of translation both in vitro and in vivo (7-9) by interacting with components of the translational machinery, such as eIF4A, eIF4B, and PABP (7,10). We recently found that only specific genes are down-regulated in BC200 RNA-knockdown HeLa cells (11), suggesting that BC200 RNA-mediated translation inhibition is controlled in the cell. However, we know relatively little about how this process is regulated. Since the cellular localization of BC200 RNA could potentially regulate its cellular function, we set out to determine the cellular localization of BC200 RNA in HeLa cervical cancer cells. We previously showed that the human monoclonal antibody, MabBC200-A3, recognizes a domain of BC200 (nts 63-107) in a structure- and sequence-dependent manner (Fig. 1A) (12). The BC200 RNA concentration-dependent immunoanalytical signals of MabBC200-A3 were found to coincide with the corresponding conventional hybridization signals (12). Here, we first confirmed that MabBC200-A3 can be used to immunostain BC200 RNA in HeLa cells, and then used it to study the cellular localization of BC200 RNA in these cells. We found that the antibody yielded concentration-dependent immunostaining signals for BC200 RNA in the tested cell line, and that BC200 RNA was localized as punctuates in both the cytoplasm and nucleus of HeLa cells.

The binding of proteins to BC200 RNA could play an important role in its subcellular localization. Recently, we identified heterologous nuclear ribonucleoprotein E2 (hnRNP E2) as a binding partner of BC200 RNA, as

assessed using a yeast three-hybrid assay (13). hnRNP E2 is a multifunctional protein that participates in a variety of cellular processes, including RNA metabolism (14, 15) and translational enhancement (16). Although it is mainly located in the nucleus, a considerable portion of hnRNP E2 is found in the cytoplasm; there, it is enriched in p-bodies and stress granules, where RNA-processing factors function to control RNA metabolism (17). Since hnRNP E2 is a constituent of p-bodies, we suspected that BC200 RNA might be localized to p-bodies through its binding to hnRNP E2. Indeed, our immunostaining analysis with MabBC200-A3 showed that BC200 RNA and hnRNP E2 colocalized along with the p-body decapping enzyme, DCP1A.

## MATERIALS AND METHODS

### Cell culture and transfection

HeLa cells were maintained in Dulbecco's modified Eagle's medium (Gibco) containing 10% fetal bovine serum (Gibco) and 1% antibiotic-antimycotic (Gibco) at 37℃ with 5% $CO_2$. For transfections, cells were seeded and incubated for 24 h, and plasmid DNA transfections were performed using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol.

### Plasmids

For expression of hnRNP E2-eCFP, the hnRNP E2-encoding sequence was PCR amplified from a human cDNA library and cloned into the *Hind*III/*BamH*I sites of pECFP(n1) (Clontech). For expression of the DCP1A-eGFP and DCP1A-mCherry fusion proteins, the DCP1A-encoding sequence was PCR amplified and cloned into the *Xho*I/*Sac*II sites of pEGFP(c1) (Clontech) or the HindIII/BamHI sites of pmCherry(c1) (Clontech). For ectopic expression of BC200 RNA, the BC200 RNA-encoding sequence was PCR amplified and cloned into pSUPER (Clonetech) to generate pSUPER-BC200.

### RNA pull-down assay

Cells were washed, suspended in 10% FBS/PBS, permeabilized by 0.1% PBST, and incubated with MabBC200-A3 (5 μg) for 30 min at room temperature. MabBC200-A3 was prepared as previously described (12). The cells were washed twice with cold PBS and lysed in cold RIPA buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% Na-deoxycholate (m/v), 0.1% SDS (w/v), 1% Triton X-100 (v/v), 2 mM EDTA]. The cell lysates were incubated with protein G agarose beads (Invitrogen) for 1 h at room temperature. The beads were collected and washed three times with cold RIPA buffer, and the immunoprecipitated RNAs were purified by phenol extraction and examined by Northern blotting. As an input control, whole-cell lysates were prepared in parallel without any antibody.

### Northern blot analysis

Total RNA was fractionated on a 6% polyacrylamide gel containing 7 M urea and transferred to a Hybond-XL membrane (GE Healthcare). Antisense oligonucleotides were 5′ end labeled with [$\gamma$-$^{32}$P] ATP (PerkinElmer Life Sciences) by T4 polynucleotide kinase (Enzynomics) and used as probes for hybridization. Hybridization was carried out in Rapid-Hyb buffer (GE Healthcare), with samples incubated at 40°C overnight (for BC200 RNA)

or at 42°C for 1 h (for the 5S RNA). The membrane was washed twice (20 min each time) at 25°C in 2× SSC buffer (20 mM sodium phosphate, pH 7.4, 0.3 M NaCl, 2 mM EDTA) containing 0.1% SDS, and twice (20 min each time) in 0.2× SSC buffer containing 0.1% SDS. The membrane was exposed to an imaging plate (Fuji BAS-IP), and the results were analyzed on a phospho-image analyzer (Fuji FLA-7000).

**Confocal laser scanning microscopy**

HeLa cells were seeded on sterilized cover slips (Marienfeld) in a 24-well plate (100,000 cells/well) and transfected with or without fluorescent fusion protein-encoding plasmids. For immunostaining, cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and then incubated with 1% BSA to block non-specific binding. The MabBC200-A3 antibody (500 ng/100 uL) was used to stain BC200 RNA, and then visualized using Cy™2 AffiniPure Donkey Anti-Human IgG (1:200, Jackson Immunoresearch). Imaging was performed using a confocal Zeiss microscope (Zeiss LSM780).

**RESULTS AND DISCUSSION**

To investigate the localization of BC200 RNA, we first examined whether the antibody, MabBC200-A3 (12), could immunostain BC200 RNA in HeLa cells. When total cell lysates were treated with the antibody, about half of the cellular BC200 RNA molecules were immunoprecipitated by the antibody (Fig. 1B), suggesting that the antibody can effectively recognize BC200 RNA in the cell. However, about 50% of the BC200 RNA molecules were not recovered by our immunoprecipitation. This may reflect that some proteins capable of interacting with the MabBC200-A3 binding motif of BC200 RNA (nts 63-107) compete with the antibody for RNA binding (12), enabling some BC200 RNA molecules to avoid interacting with the antibody.

Next, we immunostained the cellular BC200 RNA and subjected the cells to confocal fluorescence microscopy. When permeabilized cells were treated with increasing amounts of MabBC200-A3, we found that the fluorescent signal increased dose-dependently up to 1 μg (Fig. 1C). To examine whether this saturation point reflected that there was a limited amount of cellular BC200 RNA available for antibody binding, we transfected HeLa cells with increasing amounts of a BC200 RNA-expressing plasmid, pSUPER-BC200, and examined whether the fluorescent signal increased with the amount of cellular BC200 RNA. Indeed, we found that the transfected cells showed dose-dependent increases in the fluorescent signal (Fig. 2A and B), and the signal was proportional to the cellular content of BC200 RNA (Fig. 2C). Finally, we used the validated antibody to investigate the subcellular localization of BC200 RNA in further detail. We observed a dispersed fluorescence throughout the cells, including both punctate staining in the nuclei and fine punctates throughout the cytoplasm (Fig. 3A).

We recently identified heterogeneous ribonucleoprotein E2 (hnRNP E2, also known as PCBP2) as a BC200 RNA-interacting protein (13). hnRNP E2 binds to BC200 RNA mainly through the unique 3' C-rich domain (nts 162-200) of BC200 RNA (Fig. 1A), which does not overlap with the antibody-binding motif. To test whether the interaction of BC200 RNA and hnRNP E2 could be detected by immunostaining with MabBC200-A3, we transfected cells with an hnRNP E2-eCFP-expressing plasmid, immunostained BC00 RNA with MabBC200-A3, and examined the distribution of BC200 RNA and the hnRNP E2-eCFP fusion protein by confocal microscopic analysis. As shown in Figure 3B, the hnRNP E2-eCFP fusion protein exhibited both dispersed and punctate staining patterns. The punctate staining pattern overlapped with that of BC200 RNA, suggesting that BC200 RNA and hnRNP E2 colocalize in HeLa cells.

Since hnRNP E2 is known to be a constituent of p-bodies (17), we used DCP1A as a p-body marker protein (18), and examined whether BC200 RNA localizes to p-bodies. When HeLa cells were transfected with a DCP1A-

mCherry fusion construct, the DCP1A fluorescence staining pattern overlapped with that of BC200 RNA (Fig. 4A). Additional transfection experiments with both hnRNP E2-eCFP and DCP1A-mCherry fusion constructs confirmed that hnRNP E2 colocalized with DCP1A (Fig. 4B).

BC200 RNA acts as a general translation inhibitor in vitro by interacting with components of the translational machinery (7-9). However, we recently found that only a limited number of genes were altered by BC200 RNA in vivo (11), suggesting that one or more mechanisms govern the specificity of BC200 RNA-mediated translation inhibition in vivo. In neuronal cells, BC200 RNA is normally restricted to dendrites, and thus acts on dendritic mRNAs (19). In cancer cells, such as HeLa cells, BC200 RNA may also affect specifically localized mRNAs. Thus, our present observation that BC200 RNA is localized in p-bodies may suggest that BC200 RNA inhibits the translation of mRNAs localized in p-bodies. As BC200 RNA is known to be targeted to dendrites by hnRNP A2/B1 in neuronal cells (19) and the p-body constituent, hnRNP E2, is a binding partner of BC200 RNA (13), it seems likely that BC200 RNA could be targeted to p-bodies by hnRNP E2 in HeLa cells.

Although the cellular localization of BC200 RNA can be visualized by RNA fluorescence in situ hybridization (FISH) using antisense nucleic acids (20), the use of MabBC200-A3 has three major advantages over FISH. 1) Since both the sequence and structure of an RNA are crucial for the antibody-RNA interaction, the recognition of an RNA by an antibody is more specific than a complementarity-based RNA interaction, which is generally associated with nonspecific signals. 2) Whereas antisense nucleic acids are used under conditions that prevent the retention of structure, an antibody can be used to monitor complex and dynamic behaviors of RNAs that have various conformations and are involved in the formation of different ribonucleoproteins. 3) FISH is a multistep procedure that generally involves cell fixation and denaturation of target RNA (20), making it difficult (if not impossible) to detect RNA dynamics over time in living cells.

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**FIGURE LEGENDS**

Figure 1. Specific recognition of BC200 RNA by the antibody, MabBC200-A3, in HeLa cells. (A) Possible secondary structures of BC200 RNA. The blue-shaded region is the domain recognized by the antibody MabBC200-A3. Protected regions by the MabBC200-A3 antibody are highlighted in red letters. (B) HeLa cell lysates were immunoprecipitated with MabBC200-A3. RNAs were purified from the immunoprecipitates and subjected to Northern blot analysis. Cell only, without antibody. Mab N, a negative control antibody. Mab A3, MabBC200-A3. (C) Cells treated with increasing amounts of MabBC200-A3 were incubated with Cy™2 AffiniPure Donkey Anti-Human IgG and subjected to confocal microscopy. BC200 RNA is represented by green fluorescence. DAPI was used for nuclei staining.

Figure 2. Analysis of HeLa cells expressing increasing amounts of BC200 RNA. HeLa cells were transfected with increasing amounts of the BC200 RNA-expressing plasmid (pSUPER-BC200), and subjected to fluorescent signal analysis (A), and the relative signals are presented in arbitrary units (B). (C) Total RNAs were purified from the same cells indicated by the lane number subjected to Northern blot analysis.

Figure 3. Localization of BC200 RNA and colocalization with hnRNP E2in HeLa cells. (A) HeLa cells were treated with MabBC200-A3 and incubated with Cy™2 AffiniPure Donkey Anti-Human IgG, and nuclei were stained with Hoechst 33342. BC200 RNA and nuclei were visualized as green and blue fluorescence, respectively, under confocal microscopy. (B) HeLa cells were transfected with an hnRNP E2-eCFP-expressing construct, treated with MabBC200-A3, and incubated with Cy™2 AffiniPure Donkey Anti-Human IgG. BC200 RNA and hnRNP E2-eCFP were visualized as green and blue fluorescence, respectively, under confocal microscopy. Colocalization is shown by arrows.

Figure 4. Colocalization between BC200 RNA and DCP1A, and between hnRNP E2 and DCP1A. (A) HeLa cells were transfected with a DCP1A-mCherry-expressing construct, treated with MabBC200-A3, and incubated with Cy™2 AffiniPure Donkey Anti-Human IgG. Nuclei were stained with Hoechst 33342. BC200 RNA, DCP1A-mCherry, and nuclei were visualized as green, red, and blue fluorescence, respectively, under confocal microscopy. Colocalization is shown by arrows. (B) HeLa cells were transfected with hnRNP E2-eCFP- and DCP1A-eGFP-expressing constructs, and the cells were subjected to confocal microscopic analysis. hnRNP E2-eCFP and DCP1A-eGFP were visualized as blue and green fluorescence, respectively. Colocalization is shown

by arrows.

Fig. 1

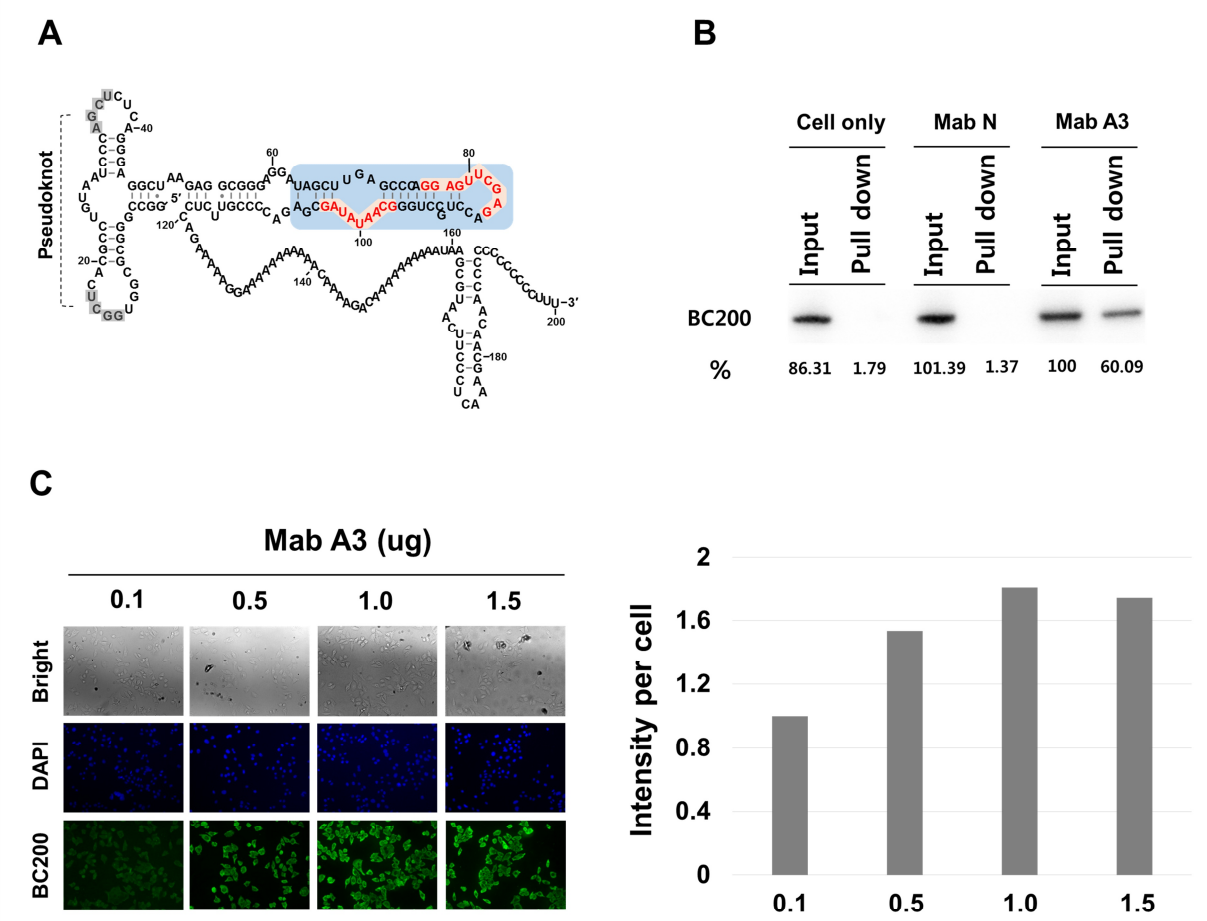
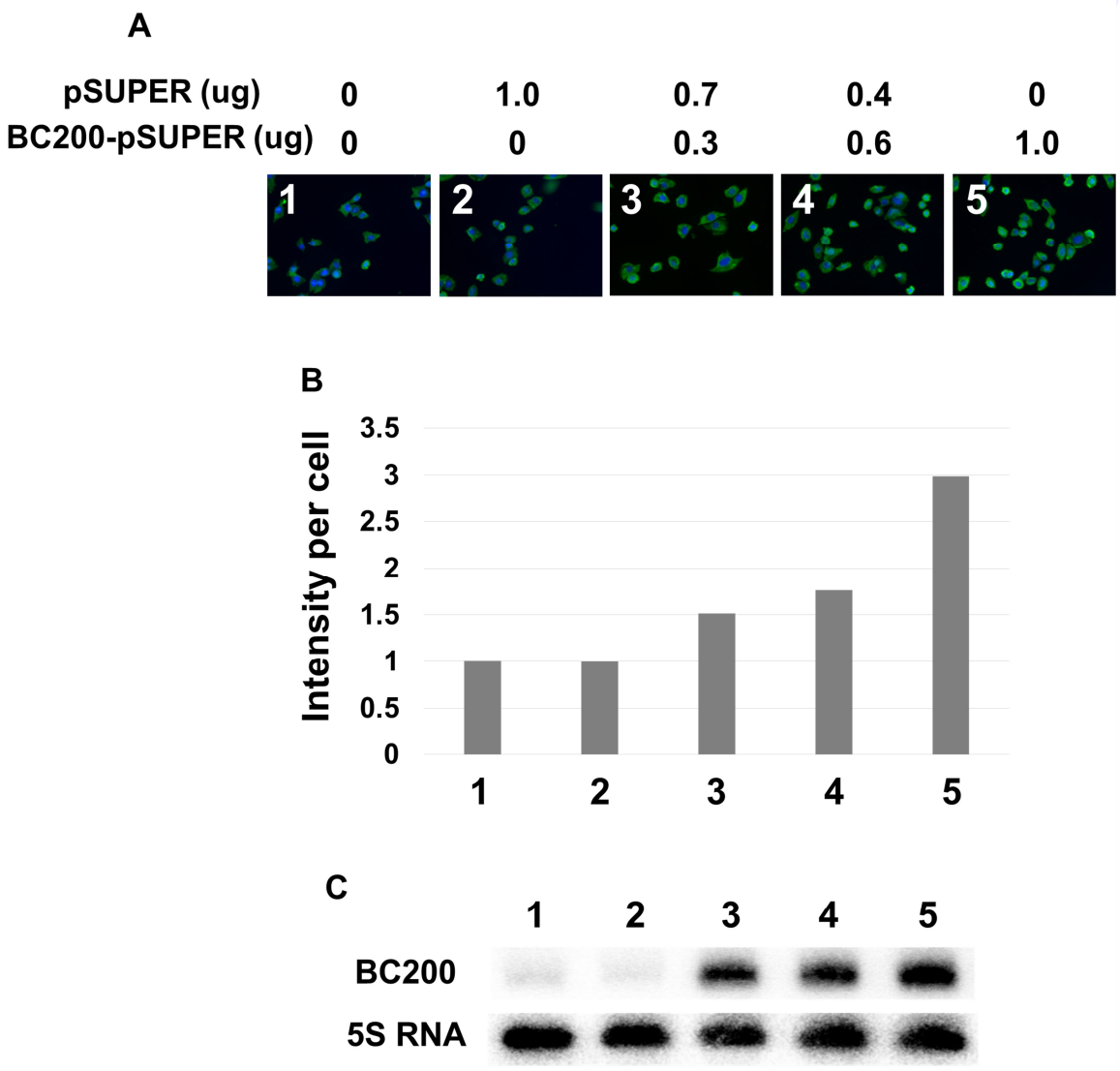


Fig. 2


A
pSUPER (ug) 0 1.0 0.7 0.4 0
BC200-pSUPER (ug) 0 0 0.3 0.6 1.0
1
2
3
4
5
B
Intensity per cell
3.5
3
2.5
2
1.5
1
0.5
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1
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3
4
5
C
1
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5
BC200
5S RNA

Fig. 3

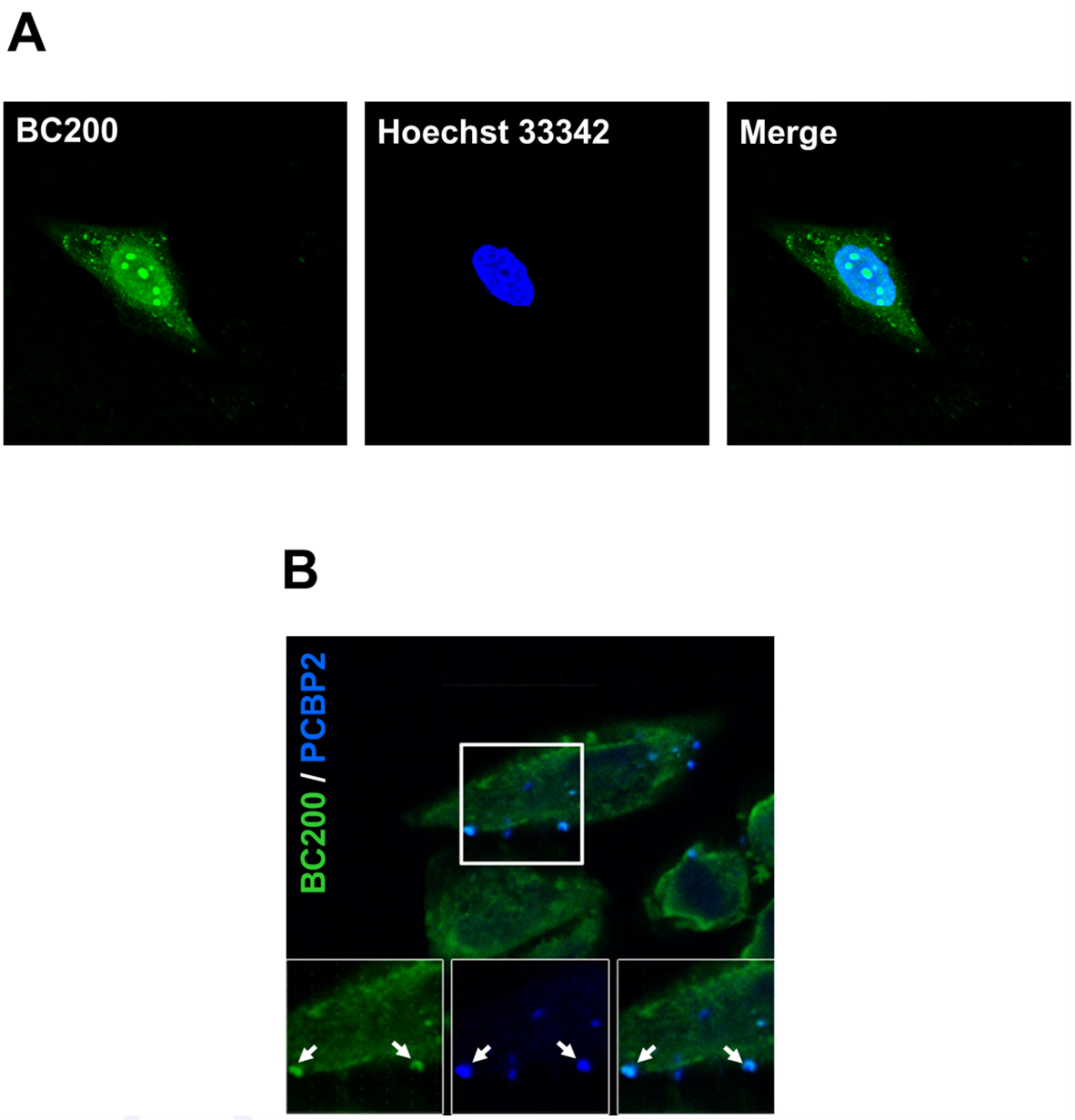

Fig. 4