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Interaction of ubiquitin ligase CBL with LMP2A protein of Epstein-Barr virus occurs via PTB domain of CBL and does not depend on adaptor ITSN1

O. V. Dergai, M. V. Dergai, D. O. Gudkova, L. O. Tsyba, I. Ya. Skrypkina, A. V. Rynditch

State Key Laboratory on Molecular and Cellular Biology
Institute of Molecular Biology and Genetics, NAS of Ukraine
150, Akademika Zabolotnogo Str., Kyiv, Ukraine, 03680

o.dergai@gmail.com

Previously Latent membrane protein 2A (LMP2A) of Epstein-Barr virus was found to be ubiquitylated by CBL ubiquitin ligase but no direct interaction of LMP2A with CBL was reported. We ***aimed*** *to explore this interaction and study a possibility of adaptor protein involvement. Taking into consideration that both LMP2A and CBL were shown to interact with endocytic adaptor protein intersectin 1 (ITSN1), we assumed that the latter could serve as a scaffold for LMP2A/CBL complex.* ***Methods****. We used an immunofluorescence and coimmunoprecipitation approaches to test a mutual complex formation of ITSN1, CBL and LMP2A proteins.* ***Results****. LMP2A coimmunoprecipitated with CBL while LMP2A did not interact with CBL G306E mutant harboring inactive phosphotyrosine-binding domain. We observed a triple colocalization of ITSN1, CBL and LMP2A signals in MCF-7 cells as well as coprecipitation of all mentioned proteins. Overexpression of ITSN1 did not affect the efficiency of complex formation of LMP2A with CBL. Moreover, LMP2A mutant unable to interact with ITSN1 was readily precipitated with CBL.* ***Conclusions****. LMP2A can be engaged in the complex together with endocytic adaptor ITSN1 and ubiquitin ligase CBL. We show that PTB domain of CBL is responsible for interaction with LMP2A. ITSN1 is not required for LMP2A recruiting to CBL.*

Keywords: intersectin 1, CBL, LMP2A, Epstein-Barr virus.

Introduction. Latent membrane protein 2A is an Epstein-Barr virus encoded transmembrane protein that is implicated in the regulation of viral latency and pathogenesis [1, 2]. LMP2A recruits a set of cellular proteins such as tyrosine protein kinases Lyn and Syk to activate Akt- and Ras-dependent signaling pathways in epithelial and B cells [3–5]. LMP2A resembles BCR (B cell receptor) by the presence of ITAM (Immunoreceptor tyrosine-based activation motif) in the N-terminal cytosolic domain [6, 7]. In contrast to BCR, LMP2A contains adjacent to ITAM several PY motifs which recruit Nedd4 family ubiquitin–protein ligases (E3s) resulting in the downregulation of LMP2A activity by ubiquitylation of LMP2A and LMP2A-associated kinases [8, 9].

Recently it has been shown that E3 ubiquitin ligase CBL ubiquitylates LMP2A and associated with LMP2A kinases, but no direct interaction between CBL and LMP2A has been reported so far [10]. Since both CBL and LMP2A are known to bind endocytic adaptor protein intersectin 1 (ITSN1) [11–13], we asked whether ITSN1 could mediate the interaction between these two proteins. ITSN1 is a multidomain adaptor protein implicated in endocytosis, regulation of signaling networks and actin cytoskeleton rearrangements [14, 15]. Due to the presence of numerous modules of protein-protein interaction ITSN1 functions as a platform to assemble multi-component complexes [16, 17].

Materials and methods. *Antibodies*. Monoclonal antibody to the FLAG epitope (clone M2) was from «Sigma» (USA); monoclonal α-Omni (D-8) antibody was from «Santa Cruz Biotechnology» (USA); a mono-

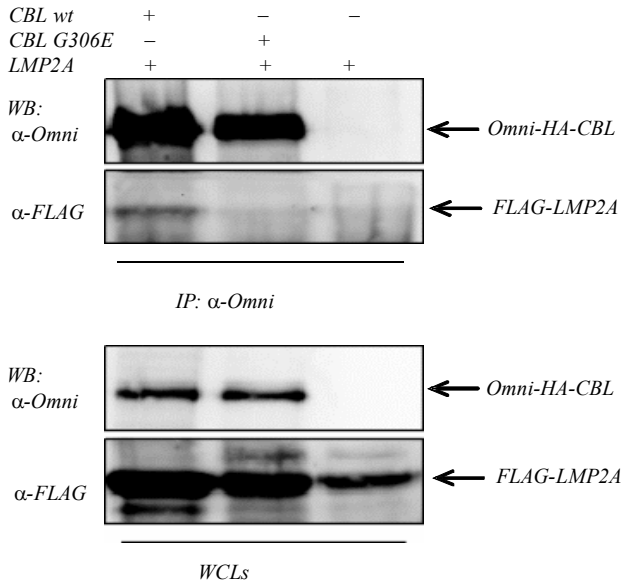

Fig. 1. PTB domain of CBL mediates the interaction with LMP2A. HEK293 cells were cotransfected with LMP2A and CBL wild-type or its mutant G306E. Posttransfection cells were harvested 24 h after transfection and cell lysates were used for CBL precipitation with α-Omni antibodies. WCLs – whole cell lysates

clonal α-HA (MMS-101P) was from Covance. Mouse α-Intersectin/ESE-1 monoclonal antibody (611574) was from «BD Transduction Laboratories» (USA). For immunofluorescence goat α-rabbit Alexa 405 («Invitrogen», USA) and horse α-mouse FITC-conjugated («Vector Laboratories», USA) antibodies were used.

Cell culture and transfection. HEK293 and MCF-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal calf serum, 50 U/ml penicillin and 100 μg/ml streptomycin. Transfections were carried out as described previously [18]. HEK293 cells stably transfected with wild type LMP2A or empty vector were generated using linearized pcDNA4/HisMax-LMP2A or pcDNA4/HisMax vector, respectively.

Transfected cells were selected on 160 μg/ml zeocin («Invitrogen») for 7 days.

Plasmids. Omni-ITSN1 was described previously [19], FLAG-LMP2A was a generous gift of Dr. G. Winberg (Karolinska Institute, Sweden), Omni-LMP2A and Omni-LMP2A YF + P5 mutant were described previously [13], mCherry-ITSN1 was described in [20]. Omni-HA-CBL wild-type and Omni-HA-tagged G306E mutant were obtained by cloning a full-length wild-type CBL or respective mutant carrying on their 5'end sequence of HA-tag, provided by Dr. H. Band (University of Nebraska, USA) in pcDNA4/HisMax C vector («Invitrogen»).

Immunoprecipitation and Western blot analysis were carried out as described previously [13].

Immunofluorescence and confocal microscopy. MCF-7 cells were plated on coverslips and transfected with plasmids encoding FLAG-LMP2A, Omni-HA-CBL and mCherry-ITSN1 using the JetPEI reagent. 24 h posttransfection cells were washed with ice-cold PBS, fixed in 4 % formaldehyde for 15 min, then washed 3 times with 1 × PBS and 0.2 % Triton X-100 for 5 min and blocked in 2 % BSA for 30 min at room temperature. The cells were incubated with the mixture of α-Omni (rabbit) and α-FLAG (mouse) primary antibody (dilution 1:100) for 1 h at room temperature. Staining with α-FLAG pAb was detected with FITC-conjugated α-mouse immunoglobulin (dilution 1:400), and α-Omni was detected with Alexa Fluor 405 conjugated α-rabbit Ig (dilution 1:400).

The slides were mounted using PVA-DABCO («Fluka», Switzerland), confocal images were taken with a Zeiss LSM510 microscope and analyzed using the LSM510 image browser software. Image of colocalized pixels were obtained using ImageJ 1.39p software, Colocalization plugin.

Results and discussion. CBL is a RING FINGER E3 ubiquitin ligase that is required for targeting substrates to the proteasomal degradation [21]. This protein contains the N-terminal phosphotyrosine binding domain (PTB) that enables its interaction with numerous tyrosine-phosphorylated substrates, catalytic RING FINGER domain, which is responsible for the ubiquitin ligase activity, multiple proline-rich motifs and C-terminal ubiquitin-associated (UBA) domain [22, 23]. The domain structure of CBL implies its association with LMP2A via PTB domain or involvement of additional adaptor protein.

The PTB domain of CBL is known to bind directly phosphotyrosines of receptors such as EGFR [22], so we assumed that this domain could mediate CBL interaction with LMP2A as well. We observed a coimmunoprecipitation of LMP2A with a wild type CBL in contrast to its G306E mutant which has PTB domain unable to interact with phosphotyrosines (Fig. 1). These findings suggest a role of PTB domain of CBL as a bin-

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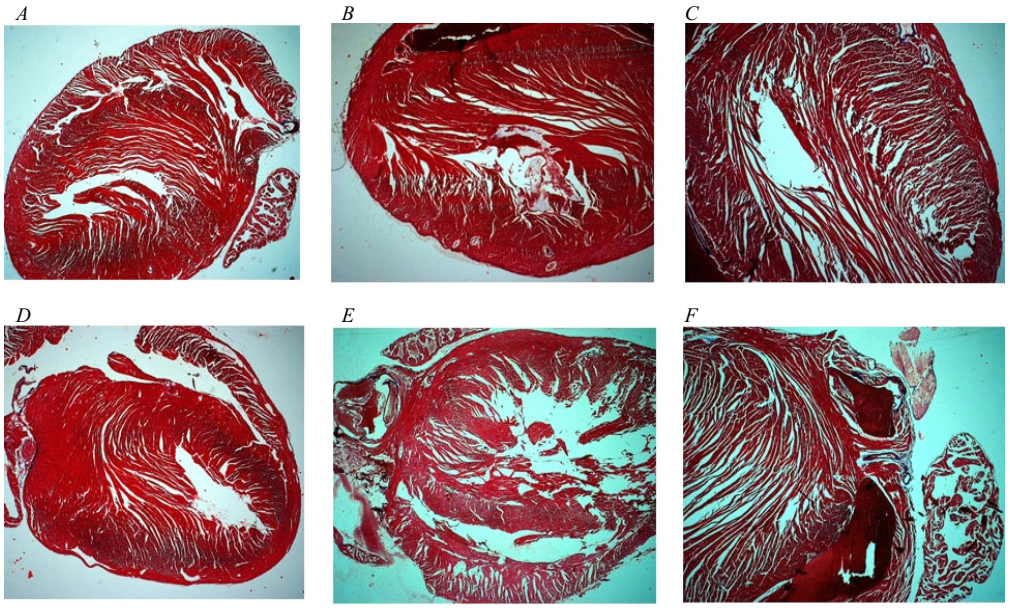

Fig. 2. Histological analysis of the -catenin haploinsufficient mice. Longitudial heart sections from -catenin flox/ WT/Cre^{+} (CKO) and -catenin flox/flox/ Cre^{-}(WT) or -catenin flox/ WT/Cre^{-} (WT) were stained using Masson's trichrome method. Analyzed mice at 1 (*A*, *D*), 3 (*B*, *E*) and 6 (*C*, *F*) months of age; WT (*A–C*), CKO (*D–F*); 4

Figure 2 to article by O. V. Dergai et al.

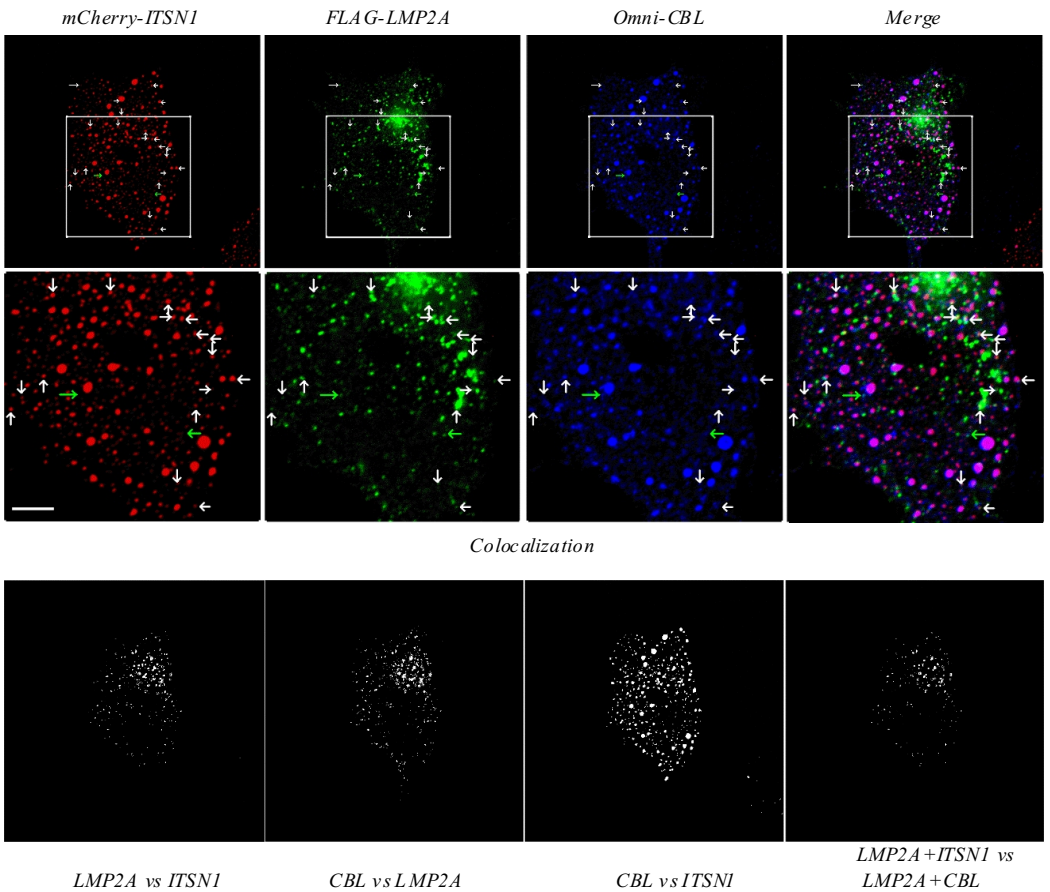

Fig. 2. ITSN1, CBL and LMP2A are colocalized in MCF-7 cells. Cells plated on coverslips were co-transfected with mCherry-ITSN1, FLAG-LMP2A and Omni-HA-CBL. 24 h post-transfection cells were fixed and processed as described in Material and methods. White arrows highlight examples of triple co-localization, green ones indicate spots of CBL and LMP2A overlapping that are ITSN1 negative. Lower panel represents colocalization of indicated proteins that was obtained from confocal images using ImageJ software. White scale bar represents 5 m

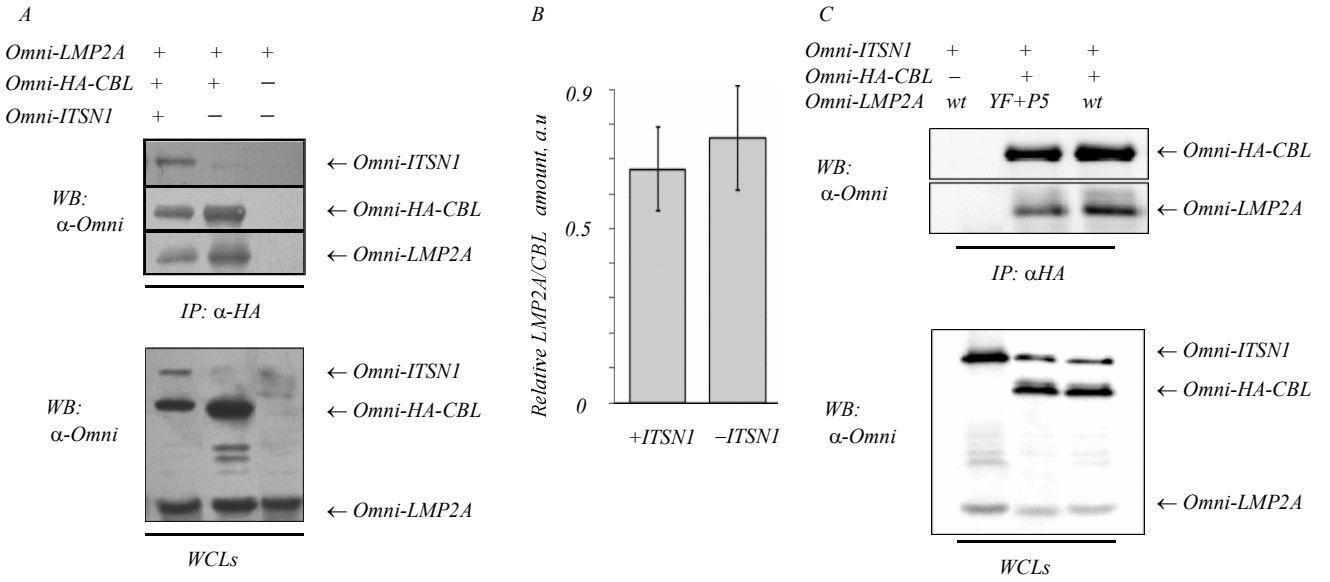

Fig. 3. ITSN1 is not required as a scaffold for LMP2A/CBL complex formation: *A* – HEK293 cells stably expressing LMP2A were cotransfected with CBL and ITSN1 or empty vector (24 h posttransfection cells were harvested and cell lysates were further applied for immunoprecipitation with α-HA antibody; precipitated proteins were eluted with standard Laemmli buffer and analyzed by Western blot analysis with indicated antibodies); *B* – diagram represents an amount of LMP2A coprecipitated with CBL normalized to CBL intensity (average means ± SD of three independent experiments are shown) C. HEK293 cells were cotransfected as indicated at the table on the top of the picture. Proteins were inmunoprecipitated with α-HA antibody and analyzed by Western blot with respective antibodies

ding site for LMP2A tyrosine-phosphorylated motifs. However, LMP2A YF + P5 mutant harboring mutations in the ITAM motif was readily coprecipitated with CBL. This data suggest that the interaction of CBL and LMP2A does not depend on phosphotyrosines of ITAM and could be mediated by other tyrosine-phosphorylated sites.

As mentioned above we can not exclude the involvement of some adaptor protein which could mediate the interaction between CBL and LMP2A. We suggested that ITSN1 could serve as such adaptor, since it was found to bind CBL [11, 12] as well as LMP2A [13] via different SH3 domains.

To address a possibility of the mutual complex formation of ITSN1, CBL and LMP2A immunoflurescence analysis was carried out. Multiple overlapping of ITSN1 and CBL signals was observed in MCF-7 cells, a certain portion of them was found to be LMP2A-positive (Fig. 2, see inset).

Almost all CBL spots were ITSN1-positive, which is in line with previous finding that ITSN1 is constitutively associated with CBL [11]. Triple colocalization supports the idea about formation of the complex containing ITSN1, CBL and LMP2A.

To study a role of ITSN1 as a potential recruiter of CBL to LMP2A, ITSN1 was overexpressed in HEK293 cells stably expressing LMP2A and transiently cotransfected with CBL. We observed the significant increase in ITSN1 amount in the complex with CBL after ITSN1 overexpression, but the amount of LMP2A coprecipitated with CBL was barely affected, $p = 0.86$ (Fig. 3, *A*, *B*). In line with this observation is the fact that a mutant of LMP2A (YF + P5) unable to bind ITSN1 [13] was readily detected in complex with CBL (Fig. 3, *C*). Taken together these results we assume that ITSN1 might be engaged in the complex of CBL and LMP2A, but it is not required to mediate this interaction. This notion can be additionally supported by observed colocalization of LMP2A and CBL signals with no overlapping with ITSN1 (Fig. 2, see inset, upper panel).

Previous attempts to detect direct or indirect interaction of CBL with LMP2A were unsuccessful [10] and reasons for this failure remain unclear. A possible explanation of this fact might be the usage of different detergents in cell lysis buffers.

CBL is shown to promote LMP2A degradation through the ubiquitylation and specifically mediates

the degradation of tyrosine kinase Syk in the presence of LMP2A. These events were found to be important for the regulation of EBV switch from the latency to the lytic phase of infection [10]. Our recent data point that ITSN1 interacts with the Syk kinase, moreover, it undergoes tyrosine phosphorylation by this kinase in LMP2A-positive cells [13]. As CBL controls Syk stability it may regulate ITSN1 phosphorylation as well. Noteworthy, CBL interacts with LMP2A via its PTB domain suggesting that this binding depends on phosphorylation of tyrosine residues of LMP2A. The interaction of CBL with LMP2A resembles its binding to a receptor molecule that occurs only when the latter has been phosphorylated. We assume that many if not all LMP2A-induced effects have self-limiting regulatory loops and the recruitment of such negative regulators as ubiquitin ligase CBL may represent an example of such control.

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О. В. Дергай, М. В. Дергай, Д. О. Гудкова, Л. О. Циба, І. Я. Скрипкіна, А. В. Риндич

Взаємодія убіквітин-лігази CBL з білком LMP2A вірусу Епштейна-Барр здійснюється за посередництвом PTB-домену та не залежить від адаптерного білка ITSN1

Резюме

Відомо, що мембранний білок латентної фази 2А вірусу Епштейна-Барр убіквітинілюється убіквітин-лігазою CBL, хоча прямої взаємодії цих двох білків не виявлено. Наша ***мета*** *полягала у дослідженні взаємодії LMP2A і CBL та вивченні можливості участі в цьому комплексі білкового адаптера. Беручи до уваги, що обидва зазначених білки взаємодіють з ендоцитозним адаптерним білком ITSN1, ми припустили, що останній може слугувати платформою для утворення комплексу LMP2A/CBL.* ***Методи****. Імунофлуоресцентний аналіз та коімунопреципітацію застосовано для дослідження можливості формування комплексу між ITSN1, CBL і LMP2A.* ***Результати****. Коімунопреципітація LMP2A і CBL свідчить про утворення комплексу цими білками, причому мутантна форма CBL, яка не здатна зв'язувати фосфотирозинові залишки, не взаємодіє з LMP2A. Ми спостерігали потрійну колокалізацію ITSN1, CBL і LMP2A у клітинах лінії MCF-7, а також коімунопреципітацію всіх зазначених білків. Надекспресія ITSN1 не впливає на ефективність коімунопреципітації LMP2A з CBL. Більш того, мутантний варіант LMP2A, не здатний зв'язуватися із ITSN1, ефективно взаємодіє з CBL.* ***Висновки****. LMP2A може входити до комплексу ендоцитозного адаптерного білка ITSN1 та убіквітин-лігази CBL. Участь ITSN1 не є необхідною для формування комплексу між LMP2A і CBL. Показано, що PTB-домен убіквітин-лігази CBL відповідає за зв'язування з LMP2A.*

Ключові слова: інтерсектин 1, CBL, LMP2A, вірус Епштейна-Барр.

А. В. Дергай, Н. В. Дергай, Д. О. Гудкова, Л. А. Цыба, И. Я. Скрипкина, А. В. Риндич

Взаимодействие убиквитин-лигазы CBL с белком LMP2A вируса Эпштейна-Барр осуществляется через PTB-домен и не зависит от адаптерного белка ITSN1

Резюме

Известно, что мембранный белок латентной фазы 2А вируса Эпштейна-Барр убиквитинилируется убиквитин-лигазой CBL, однако прямого взаимодействия этих белков ранее обнаружено не было. Наша ***цель*** *состояла в исследовании взаимодействия CBL и LMP2A, а также в изучении возможности участия в этом комплексе белкового адаптера. Принимая во внимание, что оба упомянутых белка взаимодействуют с эндоцитозным адаптерным белком ITSN1, мы предположили, что последний может служить платформой для образования комплекса LMP2A/CBL.* ***Методы.*** *Иммунофлуоресцентный анализ и коиммунопреципитацию использовали для изучения возможности образования комплекса с участием ITSN1, CBL и LMP2A.* ***Результаты****. Коиммунопреципитация CBL и LMP2A свидетельствует об образовании комплекса этими белками, причем мутантная форма CBL, лишенная способности связывать фосфотирозиновые остатки, не взаимодействует с LMP2A. Мы детектировали тройную колокализацию ITSN1, CBL и LMP2A в клетках линии MCF-7, а также коиммунопреципитацию вышеупомянутых белков. Суперэкспрессия ITSN1 не влияет на эффективность копреципитации CBL и LMP2A. Более того, мутантный вариант LMP2A, дефектный по связыванию с ITSN1, эффективно взаимодействовал с CBL.* ***Выводы****. LMP2A может включаться в комплекс эндоцитозного адаптера ITSN1 и убиквитин-лигазы CBL. Участие ITSN1 не является обязательным для образования комплекса LMP2A/CBL. Показано, что PTB-домен убиквитин-лигазы CBL отвечает за связывание с LMP2A.*

Ключевые слова: интерсектин 1, CBL, LMP2A, вирус Эпштейна-Барр.

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