J. Biochem. 2016;159(1):133–140 doi:10.1093/jb/mvv077

JB THE JOURNAL OF BIOCHEMISTRY

Characterization of Aes nuclear foci in colorectal cancer cells

Received June 11, 2015; accepted July 20, 2015; published online July 29, 2015

Yoshiro Itatani[1,2], Masahiro Sonoshita[1], Fumihiko Kakizaki[1], Katsuya Okawa[3], Stefano Stifani[4], Hideaki Itoh[5], Yoshiharu Sakai[2] and M. Mark Taketo[1,2,*]

[1]Department of Pharmacology and [2]Department of Surgery, Graduate School of Medicine, Kyoto University, Yoshida Konoé-cho, Sakyo-ku, Kyoto 606-8501, Japan; [3]Drug Discovery Research Laboratories, Kyowa Hakko Kirin Co., Ltd, 1188 Shimotogari Nagaizumi-cho, Sunto-gun, Shizuoka 411-8731, Japan; [4]Montreal Neurological Institute, McGill University, 3801 rue University, Montreal, Quebec H3A 2B4, Canada; and [5]Department of Life Science, Faculty of Engineering and Resource Science, Akita University, 1-1 Tegata Gakuen Town, Akita, 010-0852 Akita, Japan

*Makoto Mark Taketo, Department of Pharmacology, Graduate School of Medicine, Kyoto University, Yoshida Konoé-cho, Sakyo-ku, Kyoto 606-8501, Japan. Tel: +81-75-753-4477, Fax: +81-75-753-4402, email: taketo@mfour.med.kyoto-u.ac.jp

Amino-terminal enhancer of split (Aes) is a member of Groucho/Transducin-like enhancer (TLE) family. Aes is a recently found metastasis suppressor of colorectal cancer (CRC) that inhibits Notch signalling, and forms nuclear foci together with TLE1. Although some Notch-associated proteins are known to form subnuclear bodies, little is known regarding the dynamics or functions of these structures. Here, we show that Aes nuclear foci in CRC observed under an electron microscope are in a rather amorphous structure, lacking surrounding membrane. Investigation of their behaviour during the cell cycle by time-lapse cinematography showed that Aes nuclear foci dissolve during mitosis and reassemble after completion of cytokinesis. We have also found that heat shock cognate 70 (HSC70) is an essential component of Aes foci. Pharmacological inhibition of the HSC70 ATPase activity with VER155008 reduces Aes focus formation. These results provide insight into the understanding of Aes-mediated inhibition of Notch signalling.

Keywords: colorectal cancer/heat shock protein/Notch signalling/nuclear matrix/transcription.

Abbreviations: Aes, Amino-terminal enhancer of split; APC, Adenomatous polyposis coli; CK2, casein kinase II; CRC, colorectal cancer; CSK, cytoskeleton; DIG, digestion; DSP, dithiobis [succinimidylpropionate]; EM, electron microscope/microscopy; EXT, extraction; HDAC, Histone deacetylase; HSC, Heat shock cognate; MAD, matrix-associated deacetylase; MAML, Mastermind-like; NICD, Notch intracellular domain; NM-IF, nuclear matrix-intermediate filament; RBPJ, Recombination signal binding protein for immunoglobulin kappa J region; RT, room temperature; TLE, Transducin-like enhancer of split; RAMIC, Rbpj-associated molecule domain and intracellular domain of the Notch receptor.

Colorectal cancer (CRC) is caused by genetic mutations in such genes as adenomatous polyposis coli (*APC*), *KRAS*, *TP53*, *PIK3CA* and *FBXW7*, and epigenetic silencing of tumour suppressor genes (*1*). Through these genetic and epigenetic alterations, normal colonic epithelial cells turn into cancer cells; 'adenoma-carcinoma' sequence (*2*). However, our understanding is still fragmentary when it comes to how cancer metastasizes. We have previously reported that Amino-terminal enhancer of split (Aes) as a novel metastasis suppressor of CRC (*3*). Aes is a truncated member of the Groucho/transducin-like enhancer of split (TLE) family, containing only the N-terminal Q (glutamine-rich) and glycine/proline-rich (GP) domains, and lacking the C-terminal three domains present in full-length Groucho/TLE proteins (*4*). Aes is an endogenous repressor of Notch signalling transcription (*3*); genetic loss of *Aes* in the intestinal epithelium of $Apc^{+/\Delta 716}$ mice, a mouse model of familial adenomatous polyposis (*5*), causes invasion and intravasation of their intestinal tumours (*3*).

Notch signalling plays critical roles in development of both vertebrates and invertebrates (*6*, *7*). Upon binding of a Notch ligand to the Notch receptor, the latter is cleaved by an ADAM protease and γ-secretase in succession, releasing the Notch intracellular domain (NICD). NICD then moves into the nucleus, and associates with the transcription factor Recombination signal binding protein for immunoglobulin kappa J region (RBPJ) together with Mastermind-like (MAML), thereby activating transcription of several genes (*8*, *9*). Aes inhibits Notch signalling in CRC cells by converting active RBPJ transcription complex into an inactive form (*3*). Aes also forms nuclear matrix (NM)-associated foci together with TLE1, a full-length member of the Groucho/TLE family. In these foci, we can also find Notch transcription factor/cofactors RBPJ, MAML and NICD (*3*). There are some reports of Notch signalling-associated nuclear bodies in mammalian cells. For example, Notch-1 intracellular domain (N1ICD) and RBPJ are found in subnuclear bodies (*10*). Nuclear corepressor complex proteins SHARP, SKIP and SMRT also colocalize with RBPJ foci that are associated with NM and histone deacetylases (HDACs) (called matrix-associated deacetylase (MAD) body) (*11–14*). However, there is limited characterization of these Notch-associated nuclear foci.

Here, we have studied Aes nuclear foci by electron microscopy (EM) and time-lapse cinematography. Under an EM, Aes nuclear foci showed amorphous structure, lacking surrounding membrane. They dissolved during mitosis and reassembled after completion of cytokinesis. We have also found that phosphorylation of the serine residue 239 of TLE1 (TLE1 S239) is important for the focus formation. In

addition, heat shock cognate 70 (HSC70) is one of the essential components of Aes nuclear foci. Namely, inhibition of the HSC70 ATPase activity reduces nuclear focus formation significantly.

Materials and Methods

Cell lines, animals and reagents

HCT116, RKO and HEK293T cells were obtained from American type culture collection. The identities of these cell lines were confirmed in July 2014 by short tandem repeat (STR) analysis (Takara Bio CDM Center, Mié, Japan). $Apc^{+/\Delta 716}$ C57BL/6 mice have been described previously (*5*). Dithiobis [succinimidylpropionate] (DSP) and HSC70 inhibitor VER155008 were purchased from Pierce (Rockford, IL) and Tocris Bioscience (Bristol, UK), respectively. For transient transfection, we used Flag-tagged Aes and AU1-tagged TLE1 cDNAs placed in pCAG vector (*3*, *15*), Aes cDNA placed in pAcGFP (Clontech, Palo Alto, CA) and myc-tagged Rbpj-associated molecule domain and intracellular domain of the Notch receptor (RAMIC) (a recombinant NICD) in pEFBosneo (*16*). cDNAs of Aes truncation mutants were constructed by PCR using wild-type Aes cDNA as a template, followed by sequencing. Expression construct for TLE1(S239A) was published previously (*17*).

Time-lapse cinematography

cDNAs encoding AcGFP-tagged Aes on its N-terminus and TLE1 were introduced into HCT116 cells using Lipofectamine LTX (Invitrogen, Carlsbad, CA) 24 h before observation. Fluorescent photomicrographs were taken every 3 min under a TCS SP5 confocal microscope (Leica Microsystems, Buffalo Grove, IL), and cinematographs were constructed in QuickTime software.

Immunofluorescence analysis

Adenomas from $Apc^{+/\Delta 716}$ mice and cells in culture expressing Aes and/or TLE1 were processed and analysed as described (*3*). Antibodies sources: anti-Flag from Sigma (St Louis, MO), anti-AU1 from Covance (Princeton, NJ), anti-SC35 and anti-HSC70 from Abcam (Cambridge, UK), anti-C23 (nucleolin) from Santa Cruz (Santa Cruz, CA), anti-fibrillarin from Novus Biologicals (Littleton, CO), anti-Aes (*3*) and anti-HDAC3 from Cell Signalling Technology (Danvers, MA).

Cell fractionation

Cell fractionation was performed as reported previously (*18*, *19*) with some modifications. Precise description can be seen in Supplementary Materials and Methods.

Mass spectrometry analysis

Mass spectrometry (MS) was performed as previously described (*20*). Briefly, after protein samples were separated by sodium dodecyl sulfate-polyacrylamde gel electrophoresis (SDS-PAGE), they were visualized by silver staining and excised as protein bands. These protein bands were digested with trypsin (Promega) in a buffer containing 50 mM ammonium bicarbonate (pH 8.0) and 2% acetonitrile overnight at 37°C and subjected to matrix-assisted laser desorption/ionization time-of-flight MS using an Ultraflex TOF/TOF (Bruker Daltonics, Billerica, MA).

Electron microscopy

Cells with nuclear foci were fixed with 4% paraformaldehyde and 2% glutaraldehyde at 4°C for 2 h. After incubation with 1% osmium tetroxide at 4°C for 1 h, samples were dehydrated and embedded in epoxy resin. Sections were cut and stained with uranyl acetate and lead citrate as reported (*21*, *22*).

Luciferase reporter assay

Cells were transfected with a Notch signalling reporter construct pGa981-6 (*16*) and phRluc-CMV vector (Promega), using Lipofectamine LTX (Invitrogen). Luciferase activities were determined using the Dual-Luciferase Reporter Assay System (Promega) with a MITHORAS LB 940 (Berthold Technologies, Bad Wildbad, Germany). The activities of firefly luciferase were normalized against those of *Renilla* luciferase.

Results and Discussion

Aes nuclear foci disappear during mitosis and lack surrounding membrane

We have reported that Aes forms nuclear foci together with TLE1, one of the *Drosophila* Groucho homologues, in HCT116 and RKO human CRC cells and HEK293T cells in culture (Fig. 1A) as well as in adenoma cells of $Apc^{+/\Delta 716}$ mouse *in vivo* (Supplementary Fig. S1) (*3*). Similar focal structures have been reported in the nucleus such as splicing bodies or nucleoli (*23*). To investigate the possible relationship between these nuclear structures and Aes nuclear foci, we performed immunofluorescence studies. As shown in Fig. 1B, Aes foci were distinct from splicing bodies (containing SC35) and nucleoli (containing nucleolin and fibrillarin). Interestingly, however, each nucleolin focus always colocalized with a part of an Aes focus (Fig. 1B, middle row). Because nucleolin is a matrix attached region-binding protein (*24*), it is possible that Aes nuclear foci bind to the NM through nucleolin. We also found that Aes nuclear foci contained HDAC3 (ref. *3* and Supplementary Fig. S1). Therefore, it is conceivable that Aes foci share some characteristics with MAD bodies. Further study is required to determine how much these two foci are similar structurally and functionally.

To further characterize the structure of Aes nuclear foci, we next examined them under an EM. As shown in Fig. 1C, the foci showed rather amorphous structures, lacking surrounding membranes (* in Fig. 1C, top photos). Immuno-EM confirmed that these foci contained Aes protein (Fig. 1C, arrowheads in bottom photos). Because nuclear structures such as nucleoli and heterochromatins show dynamic behaviours during mitosis (*25*), we next investigated Aes foci throughout the cell cycle using AcGFP-tagged Aes and time-lapse cinematography. Previously, we confirmed that AcGFP-tagged Aes as well as Flag-tagged Aes formed focal structures in the nucleus as wild-type Aes did (*3*). Notably, these Aes foci showed dynamic changes during the cell cycle: dissolving during mitosis and reassembling after cytokinesis (Fig. 1D and Supplementary Movie). These dynamic features of Aes foci suggest that their physicochemical nature and subnuclear localization are regulated during the cell cycle like other cell cycle-associated proteins (see later).

Aes Q domain and TLE1 are required for Aes nuclear focus formation

When TLE1 is not expressed exogenously, overexpressed Aes alone does not form nuclear foci but distributes diffusely in the cytoplasm of cultured cells (Fig. 2A; ratio of expression plasmids used in transient transfections, TLE1:Aes = 0:1). When overexpressed with TLE1 that is a transcription cofactor and exists mainly in the nucleoplasm, Aes moves into the nucleus and can form nuclear foci, although some remain diffusely in the nucleoplasm (Fig. 2A) (*3*). Accordingly, we investigated how TLE1 helps the formation of Aes foci. Unexpectedly, we found that the frequency of Aes focus formation decreased with increasing amounts of Aes relative to that of TLE1, with the Notch signalling

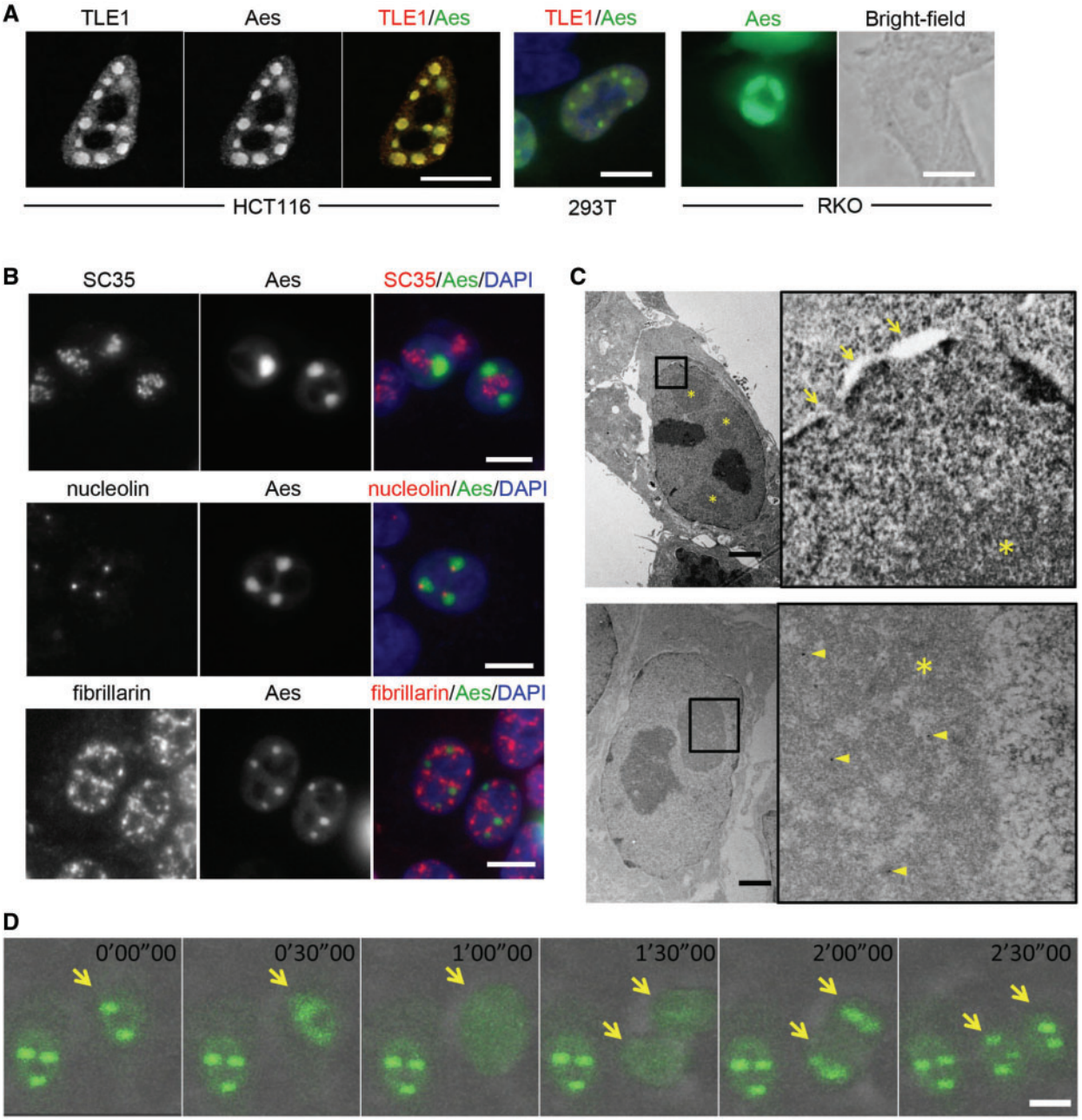

Fig. 1 Characterization of Aes nuclear foci. (**A**) AU1-tagged TLE1 (AU1-TLE1) and Flag-tagged Aes (Flag-Aes) were expressed and detected by fluorescent antibodies for each tag in HCT116 (left), HEK293T (middle) and RKO (right) cells. Note that the nuclear shape and size are different among the cell lines shown. (**B**) SC35 (a marker for splicing bodies), nucleolin and fibrillarin (both nucleolar markers, but localized to different subregions) were immunostained in HCT116 cells where AcGFP-tagged Aes (AcGFP-Aes) and TLE1 were overexpressed and immunostained simultaneously. (**C**) Flag-Aes and TLE1 were expressed in HCT116 cells and photographed by EM (upper photos). For immuno-EM (lower photos), rabbit anti-Flag and 12-nm colloidal Gold-conjugated anti-rabbit antibodies were used to localize Flag-Aes (arrowheads). Boxed areas in left panels were enlarged (right panels). Arrows, nuclear membrane; asterisk, Aes nuclear foci. (**D**) Chronological changes of Aes nuclear foci in HCT116 cells where AcGFP-Aes and TLE1 were expressed. Arrows, a cancer cell with Aes nuclear foci that went through a cell division during the recording, and its daughter cells. Scale bars, 10 μm (A, B, D), 2 μm (C).

transcription activity also decreasing concomitantly as determined with a luciferase reporter (Fig. 2A and B). Given our earlier observation that coexpression of Aes and TLE1 caused nuclear focus formation (*3*), it seemed counterintuitive that focus formation was decreased with relatively higher amounts of Aes. This may be explained that the soluble form Aes-TLE1 complex is responsible for the inhibitory effect on the Notch signalling-mediated transcription, with a fraction of Aes-TLE1 complex can be precipitated as nuclear foci. Such an interpretation is consistent with an example of toxicity caused by the soluble amyloid-beta (Aβ) oligomers rather than by its precipitates (*26*). We coin this interpretation as 'wine tannin hypothesis' because it is not the precipitated tannin, but rather the soluble form that causes an astringent taste in wine. This hypothesis may also be supported by our observation that Aes foci are completely dissolved during mitosis. It is interesting to speculate that Aes is involved in silencing Notch-dependent transcription

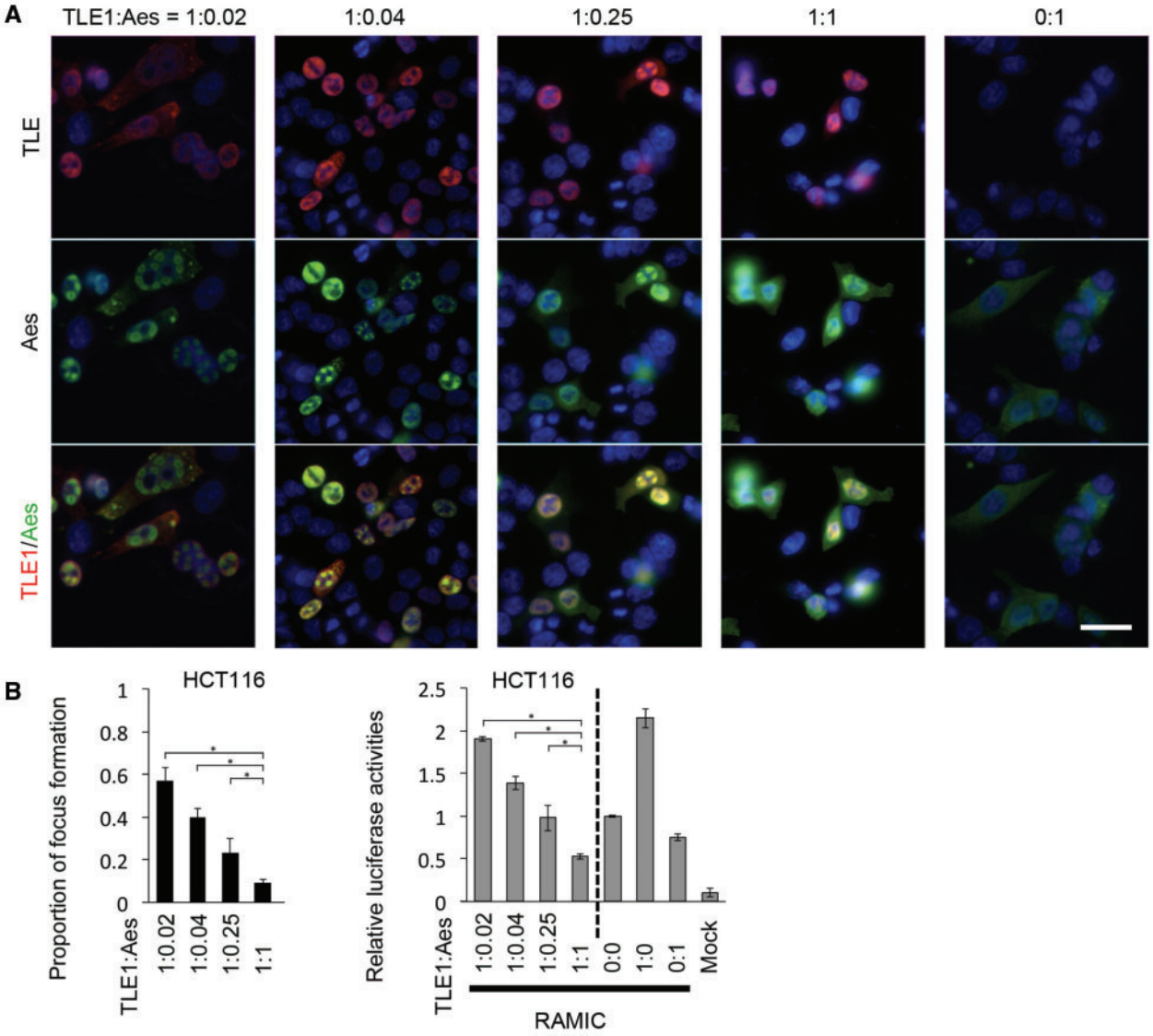

Fig. 2 Optimal focus formation in the presence of Aes and TLE1. (**A**) AU1-TLE1 and Flag-Aes were expressed in HCT116 cells at the ratios shown beneath the panels. When only Aes was expressed exogenously, focus formation could not be detected (0:1). (**B**) Upper graph shows the efficiency of Aes nuclear focus formation (focus-forming cells among the transfected cells) in HCT116 cells at the ratios of TLE1 and Aes shown in (A). Lower graph shows the analysis of Notch-mediated transcriptional activation using a luciferase reporter construct (pGa981-6; refs. *3* and *16*) in HCT116 cells transfected with Aes and TLE1. (*$P < 0.01$)

in the mitotic phase during which mRNA transcription is reduced significantly (*27*).

It is known that serine residue 239 (S239) of TLE1 is phosphorylated by casein kinase II (CK2), and its phosphorylation is important for TLE1 to repress transcription (Fig. 3A left) (*17*, *28*). Therefore, we studied whether phosphorylation of TLE1(S239) was critical for the inhibition of Notch signalling-mediated transcription and/or focus formation with Aes. To this end, we overexpressed a mutant form of TLE1 of which S239 was converted to a structurally similar but unphosphorylatable amino acid alanine (S239A) (*28*) in HCT116 CRC cells. Compared with wild-type TLE1, S239A mutant did not show inhibition of Notch signalling transcription when co-expressed with Aes (Fig. 3B). At the same time, the cells expressing TLE1(S239A) showed significantly lower frequency of Aes focus formation than those expressing the wild type (2% versus 10% of focus forming cells among the transfected cells when the equal amounts of Aes and TLE1 cDNAs were transfected. A representative photograph is shown in Fig. 3A right). These results may appear contradictory to the 'wine tannin hypothesis' if the soluble form of Aes-TLE1 complex is responsible for transcriptional inhibition. We speculate that being soluble is a necessary but not the sufficient feature for Aes-TLE1 to inhibit transcription, and that TLE1(S239A) is unable to interact with the Rbpj transcription factor complex as with other transcription factors (*17*, *28*).

To study the interaction of Aes with TLE1 further, we next constructed a pair of Aes deletion mutants each of which lacked C-terminal (N-136t; t for truncation) or N-terminal (t94-C) residues (Fig. 3C left), and exogenously expressed them with TLE1 in HCT116 CRC cells. We found that Aes(N-136t)

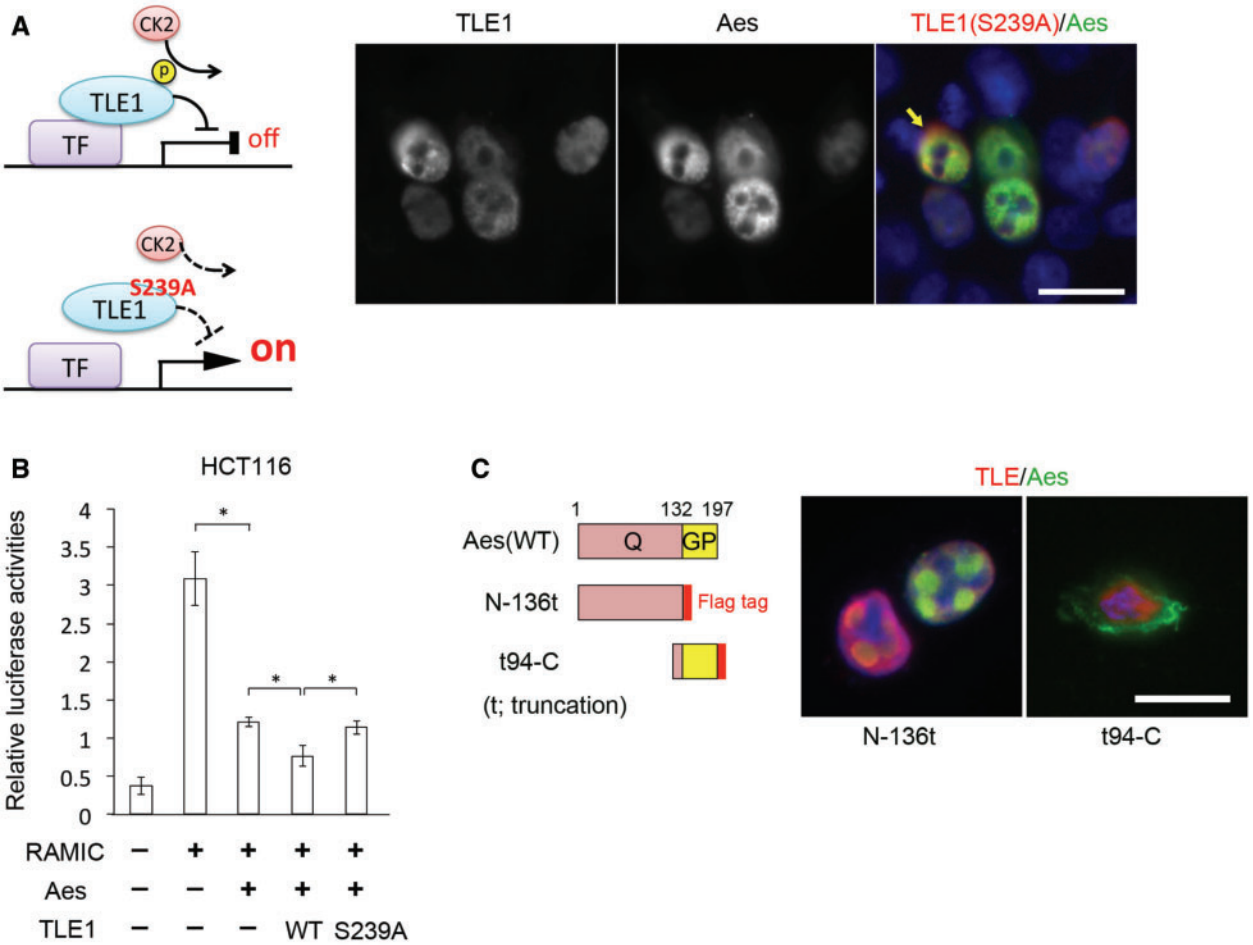

Fig. 3 Aes focus formation with mutated proteins. (**A**) Schematic representation of TLE1 transcriptional repression through phosphorylation of S239 of TLE1 (left), and an immunostaining photomicrograph when TLE1(S239A) was expressed together with AcGFP-Aes in HCT116 cells (right). TLE1(S239A) failed to form foci (~2% of focus-forming cells among all transfected cells when the same amount of AcGFP-Aes and TLE1 cDNA were transfected). The arrow points to the cell expressing both TLE1(S239A) and Aes at high levels. TF, transcription factor; CK2, casein kinase II; p, phosphorylation of TLE1 S239. (**B**) Analysis of Notch-mediated transcriptional activation using a luciferase reporter assay (pGa981-6) (*3*) in HCT116 cells transfected with Aes and TLE1 (wild type or S239A) (*$P < 0.01$). WT, wild type. Values indicate the means ± SD (**C**) Schematic drawing of Aes deletion mutants (left) and immunostaining photomicrographs when Aes mutation constructs were expressed together with TLE1 in HCT116 cells (centre and right). t, truncation. WT, wild type. Values indicate the means ± SD Scale bars, 20 μm (A, C), 10 μm (**D**).

efficiently formed nuclear foci, whereas Aes(t94-C) did not (Fig. 3C right). This result is consistent with the observation that Aes and Groucho/TLE proteins oligomerize through their Q domains (*29*). Taken together, these results suggest that formation of Aes nuclear foci requires both the N-terminal Q domain of Aes and phosphorylation of S239 in TLE1 outside of its Q-GP domains.

Heat shock cognate 70 (HSC70) is an integral component of Aes nuclear foci

We have recently reported that Aes nuclear foci are associated with insoluble NM, and contain transcription factor/cofactors involved in Notch signalling including RBPJ, MAML1 and NICD, as well as TLE1, when all of them were exogenously expressed in cultured cells (*3*). To identify additional endogenous components of Aes nuclear foci, we overexpressed Flag-Aes and TLE1 in HCT116 cells, and isolated the NM fraction. To prevent loss of the focus components during the preparation process, we used chemical cross-linker DSP prior to subcellular fractionation. With DSP cross-linking, Aes nuclear foci remained associated with the insoluble NM while residual Aes in the nucleoplasm was washed away (Fig. 4A). We then subjected the NM fraction to immunoprecipitation using anti-Flag antibody, followed by SDS-PAGE (Fig. 4B). Silver staining showed an ~70 kDa protein that was co-immunoprecipitated with Flag-Aes. A mass-spectrometry analysis identified it as HSC70, one of the heat shock proteins (HSPs). We confirmed colocalization of endogenous HSC70 with the Aes nuclear foci by immunofluorescence analysis of HCT116 cells (Fig. 4C).

HSC70 ATPase activity is essential for Aes nuclear focus formation

HSC70 is a member of the HSP70 family, and is a molecular chaperone. Although most other HSP70 family members are induced when cells are under stress such as a high temperature, HSC70 is expressed constitutively, suggesting its essential role in normal cellular physiology and homeostasis (*30*). Therefore,

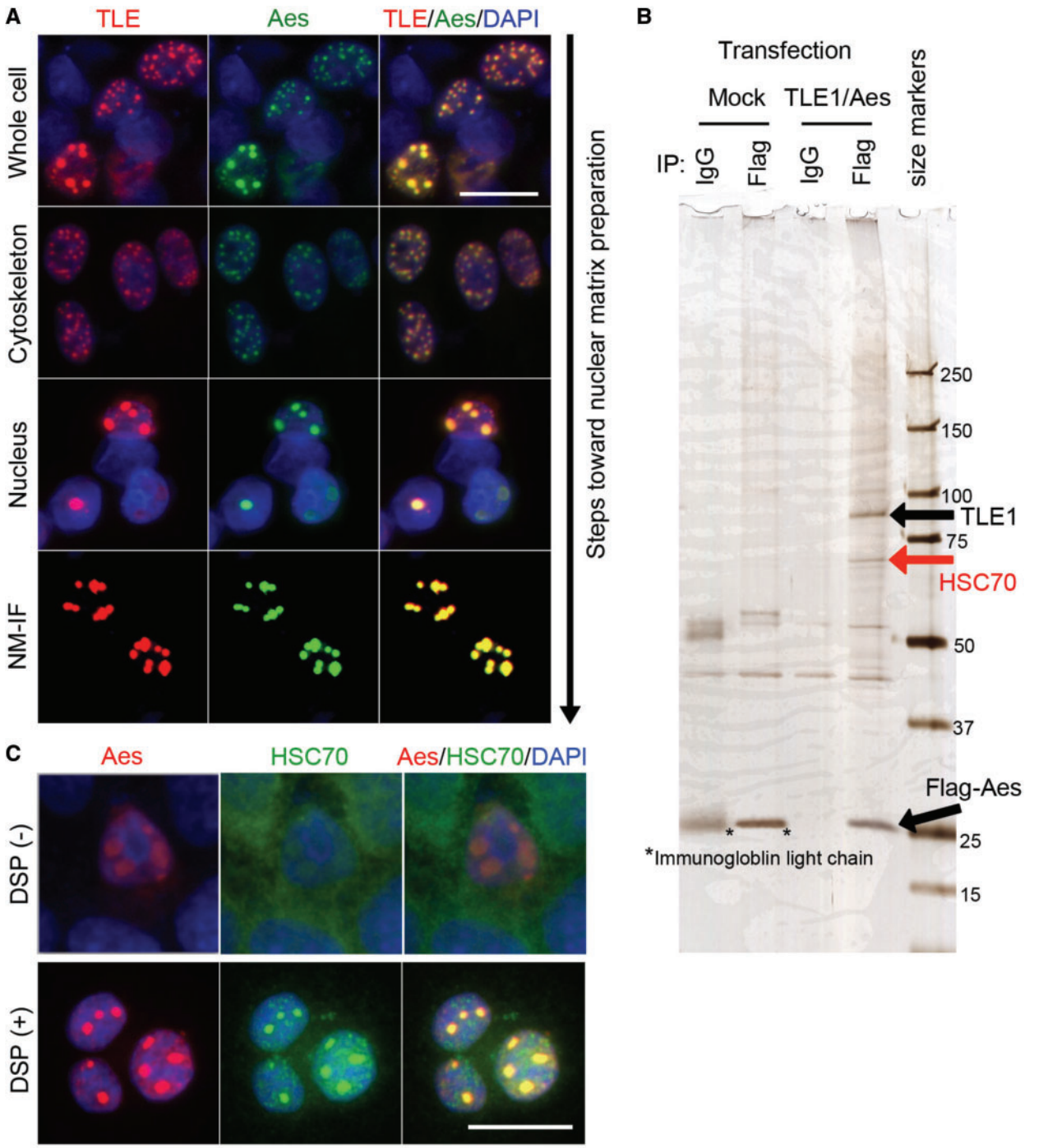

Fig. 4 HSC70 is a component of Aes foci. (**A**) Immunostaining photomicrographs of Aes nuclear foci associated with the NM. AU1-TLE1 and Flag-Aes were expressed in HCT116 cells and the cells were permeabilized and washed in cytoskeleton (CSK), EXT (extraction) and digestion (DIG) buffers as described in Supplementary Materials and Methods, followed by immunostaining. CSK, Nucleus and NM-intermediate filament (NM-IF) indicate the products obtained after washing with CSK, EXT and DIG buffers, respectively. (**B**) Silver-stained SDS-PAGE gel loaded with immunoprecipitates of NM fractions. After HCT116 cells were transfected with Flag-Aes and TLE1 or Mock empty control, and treated with 2 mM DSP, the NM fraction was prepared and immunoprecipitated with anti-Flag antibody or control IgG. Immunoprecipitates were separated by SDS-PAGE, followed by silver staining. Size markers, kDa. (**C**) HCT116 cells were transfected with Flag-Aes and TLE1 and treated with or without 2 mM DSP, followed by staining for endogenous HSC70 as well as for Flag-Aes and nuclei (DAPI). Scale bars, 20 μm.

we hypothesized that HSC70 is important for the Aes nuclear focus structure. To test this possibility, we treated HCT116 cells with VER155008, an HSC70 inhibitor that binds to the ATPase pocket of HSC70 to inhibit its function as a molecular chaperone (*31*). Notably, VER155008 inhibited nuclear focus formation in a dose-dependent manner without affecting cell viability (Fig. 5A and B). These results suggest that HSC70 ATPase activity is necessary for Aes-TLE focus formation and/or maintenance.

We also evaluated the effects of VER155008 on Notch signalling transcription. Together with TLE1,

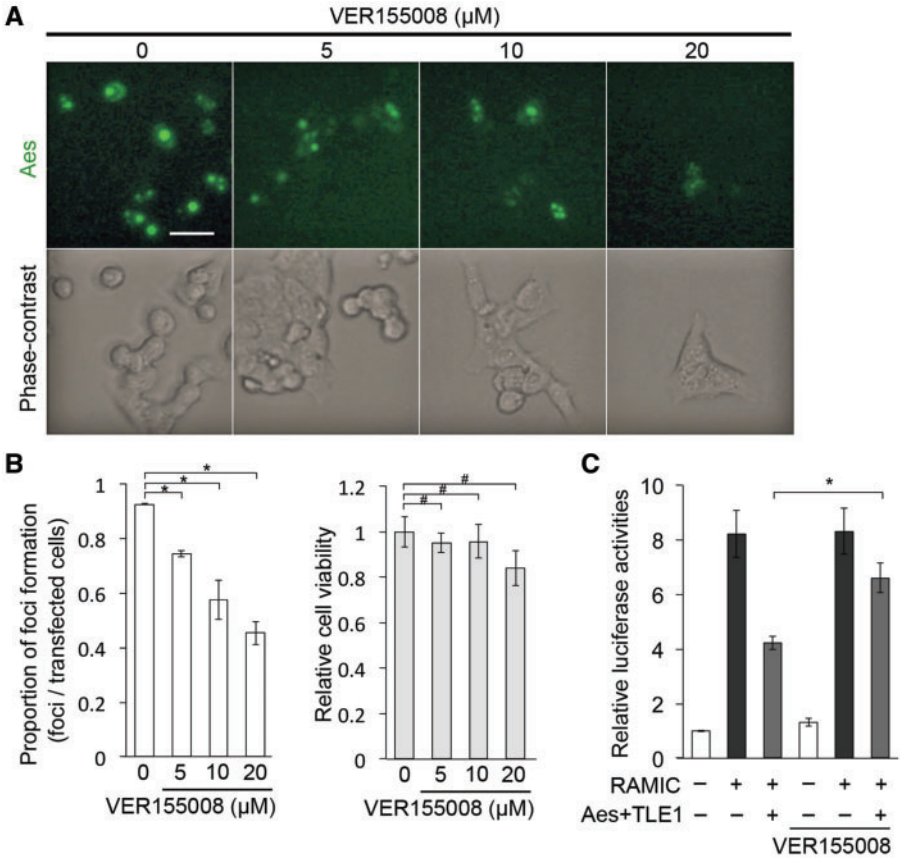

Fig. 5 ATPase activity of HSC70 is important for Aes nuclear focus formation. (**A**) Effects of HSC70 inhibitor VER155008 on the formation of Aes nuclear foci. HCT116 cells were transfected with AcGFP-Aes and TLE1 followed by the treatment with VER155008 at the indicated concentrations for 24 h. Shown are immunofluorescence photomicrographs (top row) and phase-contrast photomicrographs (bottom row). (**B**) Proportion of Aes nuclear foci formed in the presence of VER155008 quantified from the data shown in (A) (left), and the cell viability assessed with the WST-1 assay when HCT116 cells were treated with VER155008 at the indicated concentrations for 24 h (right). $*P<0.01$, $\#P>0.05$. (**C**) Analysis of Notch-mediated transcriptional activation using a luciferase reporter assay (pGa981-6) (*3*) in HCT116 cells transfected with Aes and TLE1, either in the absence or presence of VER155008 for 24 h (10 μM) ($*P<0.01$). Values indicate the means ± SD Scale bar, 20 μm.

Aes inhibited Notch transcriptional activation by ~50% in transfected cells mediated by RAMIC. Treatment with VER155008 suppressed this inhibitory effect by Aes and TLE1 slightly (Fig. 5C). Based on our wine tannin hypothesis that soluble form of Aes-TLE1 complex is responsible for inhibition of Rbpj-mediated transcription, reduction in the focus-associated fraction of Aes by VER155008 should increase the soluble Aes-TLE1 and therefore reduce the luciferase reporter activity; in fact it was ~50% higher than that without VER15508 (Fig. 5C). Accordingly, the apparent suppression by VER155008 of the inhibition of Notch signalling transcription in the presence of Aes may be some indirect effects, and needs further investigation.

In conclusion, we interpret these results that HSC70 is an integral component of Aes nuclear foci, and its ATPase activity is important for the formation and/or maintenance of the Aes foci, although its role in Notch signalling-mediated transcription requires further study.

Supplementary Data

Supplementary Data are available at *JB* Online.

Acknowledgements

The authors thank T. Honjo for plasmids and suggestions, M. Okabe for plasmids, R. Sasaki for cinematography and K. Okamoto-Furuta for electron microscopy.

Funding

This work was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan to M.S. and M.M.T.

Conflict of Interest

None declared.

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