The COP9 signalosome controls ubiquitinylation of ABCA1

Yuya Azuma [a], Mie Takada [a], Minami Maeda [a], Noriyuki Kioka [a], Kazumitsu Ueda [a,b,*]

[a] Laboratory of Cellular Biochemistry, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
[b] Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Kitashirakawa-oiwake-cho, Kyoto 606-8502, Japan

ARTICLE INFO

ABSTRACT

ATP-binding cassette protein A1 (ABCA1) mediates the transfer of cellular free cholesterol and phospholipids to apolipoprotein A-I (apoA-I), an extracellular acceptor in plasma, to form high-density lipoprotein (HDL). ABCA1 has been suggested to be degraded by proteasome in cholesterol-loaded macrophages, however, the mechanism and regulation of proteasomal degradation of ABCA1 remain unclear. In this study, we analyzed the putative interaction between ABCA1 and COP9 signalosome (CSN), a key molecule in controlling protein ubiquitination and deubiquitination. CSN2 and CSN5, subunits of COP9 CSN complex, were coprecipitated with ABCA1 when coexpressed in HEK293 cells and proteasomal degradation was inhibited by MG132. Overexpression of CSN2 increased endogenous CSN7 and CSN8, and decreased ubiquitinylated forms of ABCA1. These results suggest that CSN is a key molecule which controls the ubiquitinylation and deubiquitinylation of ABCA1, and is thus an important target for developing potential drugs to prevent atherosclerosis.

© 2009 Elsevier Inc. All rights reserved.

Introduction

The maintenance of cellular cholesterol homeostasis is important for normal human physiology; its disruption can lead to a variety of pathological conditions, including cardiovascular disease [1]. ATP-binding cassette protein A1 (ABCA1), a key factor in cholesterol homeostasis, mediates the secretion of cellular free cholesterol and phospholipids to an extracellular acceptor, apolipoprotein A-I (apoA-I), to form high-density lipoprotein (HDL) [2,3]. HDL formation is the only known pathway for the elimination of excess cholesterol from peripheral cells. Defects in ABCA1 cause Tangier disease [4–6], in which patients have a near absence of circulating HDL, prominent cholesterol–ester accumulation in tissue macrophages, and premature atherosclerotic vascular disease [1,7].

Because cholesterol is an essential component of cells, however, excessive elimination of cholesterol could result in cell death, and ABCA1-mediated cholesterol efflux is highly regulated at both the transcriptional and post-transcriptional levels. The degradation of ABCA1 is regulated [8–10] and is carried out via several pathways; (i) cell-surface ABCA1 is endocytosed and recycled back to the plasma membrane or delivered to the lysosomes through early and late endosomes for degradation [11,12], (ii) calpain protease degrades ABCA1 on the plasma membrane [13,14] and intracellularly, espe-

cially when apoA-I does not bind to ABCA1 [15]. ABCA1 is also degraded through the ubiquitin–proteasome pathway [16], however, the mechanism and regulation of ABCA1 through the ubiquitin–proteasome pathway is still unclear, although it is supposed to be important in free cholesterol-loaded macrophages.

Recently, it was reported that COP9 signalosome (CSN) complex plays an important role in the ubiquitinylation and deubiquitinylation of various proteins, such as IκBα [17]. The CSN complex consists of eight subunits (CSN1–CSN8), and CSN5 and CSN6 subunits are active centers of dissociation of the ubiquitin like protein Nedd8 from cullin [18]. Because the attachment of Nedd8 (neddylation) to cullin is essential to stimulate recruitment of an ubiquitin-loaded E2 enzyme to cullin-based ubiquitin E3 ligase, this deneddylation activity of CSN is necessary for normal regulation of cullin-based ubiquitin ligase activity. Other subunits, such as CSN1, CSN2, and CSN4, were reported to enhance CSN assembly and stabilize the CSN complex [19–21]. In this study, we analyzed a putative interaction between ABCA1 and CSN subunits. Our results suggest that the CSN complex associates with ABCA1 and controls ubiquitinylation and deubiquitinylation of ABCA1.

Materials and methods

Materials. We generated a mouse monoclonal anti-ABCA1 antibody, KM3110, which recognizes the C-terminal 20 amino acids of human ABCA1 [9]. Monoclonal mouse anti-Flag antibody (M-2) and monoclonal mouse anti-ubiquitin antibody (P4D1) were purchased from Santa Cruz Biotechnology (CA, USA). Polyclonal rabbit anti-CSN7 and anti-CSN8 were purchased from Affiniti/Biomol (PA,

USA). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and anti-rabbit IgG were purchased from Bio-Rad (CA, USA). Protein G-sepharose was purchased from Sigma–Aldrich (Tokyo, Japan). MG132 was purchased from Calbiochem (WI, USA). TO901317 was purchased from Cayman Chemical (MI, USA). Acetyl-LDL was purchased from Biomedical Technologies (MA, USA). Other chemicals were purchased from Sigma (MO, USA), GE Healthcare Biosciences (NJ, USA), Wako Pure Chemical Industries (Osaka, Japan), or Nacalai Tesque (Kyoto, Japan).

DNA construction. We generated expression constructs for human CSN2 and CSN5 by cloning PCR-amplified full-length cDNA into the expression vector pcDNA3.1. In addition, a sequence encoding the Flag tag was fused to the 5′ terminal of the CSN sequence. pcDNA3.1-ABCA1 [9] was used for the expression of ABCA1.

Cell culture and transfection. Human embryonic kidney (HEK)-293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37 °C in 5% CO_2. They were continuously kept in a subconfluent state. Transient transfection was performed using LipofectAMINE Plus Reagent (Invitrogen). To obtain mouse peritoneal macrophage cells, C57Black6/J female mice (10 weeks old) were injected with 2.0 ml PBS containing 4.0% thioglycollate intraperitoneally, and the macrophages were harvested 3 days later by peritoneal lavage. The harvested cells were plated in cell culture plates in RPMI1640 supplemented with 10% fetal bovine serum. Twenty-four hours after plating, the adherent macrophages were incubated with 50 μg/ml acetyl-LDL and 3 μM TO901317 for 24 h to induce the expression of ABCA1. Animal experiments were conducted in accordance with institutional policies following approval from the Animal Experimentation Committee of the Graduate School of Agriculture, Kyoto University.
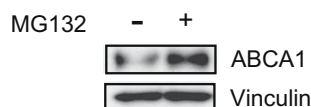
Immunoprecipitation. Thirty–fifty percent confluent HEK293 cells were transiently cotransfected with pcDNA3.1-ABCA1 and pcDNA3.1-CSN. After 24 h, cells were washed with serum-free growth medium and incubated with serum-free growth medium containing 0.02% bovine serum albumin (BSA) in the presence or absence of 50 μM MG132 for 4 h. Cells were washed with PBS and lysed in ice-cold PBS containing 1% Nonidet P-40 and protease inhibitors, 100 μg/ml (*p*-amidinophenyl)methanesulfonyl fluoride, 2 μg/ml leupeptin, and 2 μg/ml aprotinin. Cell lysates were incubated with 5 μg anti-Flag antibody M2 for 1 h at 4 °C. The immunocomplexes were incubated with protein G-sepharose for 1 h and washed four times with lysis buffer. The bound proteins were separated by SDS–PAGE (7–15%) and analyzed by immunoblotting using the antibodies. Western blotting bands were quantified using a Fujifilm LAS-3000 imaging system.

Results

Proteasomal degradation of ABCA1 in cholesterol-loaded macrophages

When mice peritoneal macrophages, loaded with cholesterol with acetyl-LDL, were treated with 20 μM MG132, a proteasome inhibitor, for 4 h, the amount of ABCA1 increased 1.8-fold compared to without treatment (Fig. 1). This was consistent with previous reports which showed increased ABCA1 in cholesterol-loaded macrophages by lactacystin treatment [14]. These results suggested that ABCA1 is degraded by proteasome in cholesterol-loaded macrophages.

CSN complex associates with ABCA1

As the first step, to reveal the mechanism and regulation of proteasomal degradation of ABCA1, we analyzed the putative interaction between ABCA1 and CSN, a key molecule in controlling protein ubiquitination and deubiquitination. HEK293 cells were transiently transfected with expression vectors for ABCA1 and Flag-tagged CSN5, a subunit of the CSN complex, and ABCA1 was immunoprecipitated with anti-ABCA1 antibody. As shown in Fig. 2A, Flag-tagged CSN5 was coimmunoprecipitated with ABCA1 in the presence of MG132, but not in its absence. The association of ABCA1 and Flag-tagged CSN5 in the presence of MG132 was confirmed by their coprecipitation with anti-Flag antibody (Fig. 2B).

It was reported that the CSN5 subunit, not as a subunit of the CSN complex, independently interacts with the target protein,

Fig. 1. Proteasomal degradation of ABCA1 in macrophages. Peritoneal macrophages from C57 mice were incubated with 50 μg/ml acetyl-LDL and 3 μM TO901317 for 24 h to induce ABCA1 expression. The macrophages were incubated with or without 20 μM MG132 for 4 h. Cell lysates were subjected to immunoblotting using antibodies against ABCA1 and vinculin. Vinculin was used as a loading control.
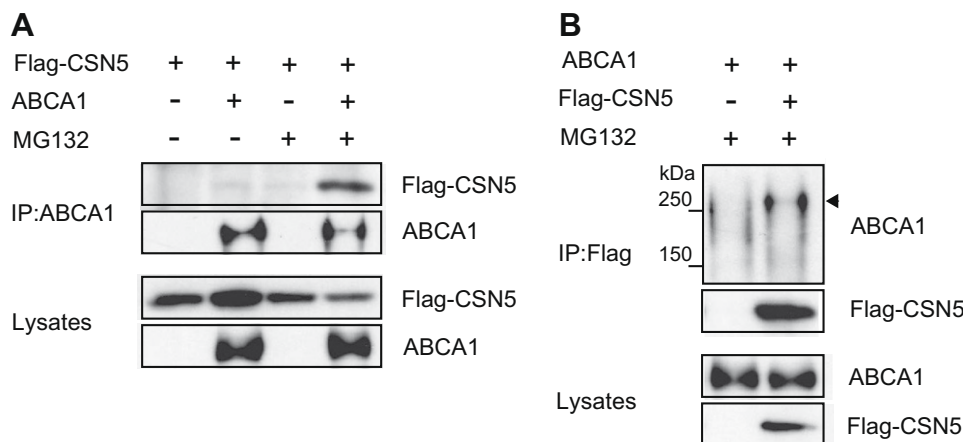
Fig. 2. CSN5 associates with ABCA1 in the presence of proteasome inhibitor. (A) HEK293 cells were transiently cotransfected with ABCA1 and Flag-CSN5. Twenty-four hours after transfection, cells were incubated with or without 50 μM MG132 for 4 h. Cell lysates were immunoprecipitated (IP) with anti-ABCA1 antibody. Immunocomplex and cell lysates were subjected to immunoblotting using antibodies against Flag and ABCA1. (B) Cell lysates from cells treated with MG132 were immunoprecipitated with anti-Flag antibody. Immunocomplex and cell lysates were subjected to immunoblotting using antibodies against ABCA1 and Flag.

such as integrin LFA-1 and DNA topoisomerase IIα, to modulate their functions [22,23]. To confirm that ABCA1 is associated with the CSN complex, the interaction of ABCA1 with CSN2, another subunit of the CSN complex, was examined. When HEK293 cells were transiently transfected with expression vectors for ABCA1 and Flag-tagged CSN2, Flag-tagged CSN2 was coimmunoprecipitated with ABCA1 in the presence of MG132, but not in its absence (Fig. 3A). It has been reported that CSN2 enhances CSN assembly and stabilizes the CSN complex [19–21]. Indeed, overexpression of Flag-tagged CSN2 in HEK293 cells increased the amounts of endogenous CSN7 and CSN8 by 3.1- and 2.8-fold, respectively (Fig. 3B). These results suggest that CSN2 enhances CSN assembly and stabilizes the CSN complex in HEK293 cells and probably that the CSN complex associates with ABCA1 in ubiquitinylated forms.

CSN promotes deubiquitinylation of ABCA1

It has been shown that interaction with CSN increases the stability of IκBα via the inactivation of Cullin1-based E3 ligase and the promotion of deubiquitinylation by CSN-associated deubiquitinylase Ubp12/USP15 [17]. We expected that the association with the CSN complex would reduce ubiquitinylated ABCA1 via similar pathways. When HEK293 cells expressing ABCA1 were treated with MG132, ubiquitinylated forms of ABCA1 were increased 2.4-fold (Fig. 4), however, when Flag-tagged CSN2 was coexpressed, ubiquitinylated forms of ABCA1 did not increase in the presence of MG132 and remained at the basal level as before MG132 treatment. These results suggest that the association with the CSN complex decreases ubiquitinylated ABCA1.

Discussion

Our results suggest that the CSN complex is a key factor which controls the ubiquitinylation and deubiquitinylation of ABCA1 and consequently controls the proteasomal degradation of ABCA1. CSN has been reported to interact with IκBα and suppresses the degradation of IκBα through its deubiquitinylation activity, and subsequently IκBα inactivates NF-κB to inhibit the secretion of inflammatory cytokines [17]. The accumulation of excess intracellular free cholesterol causes late lesional inflammation mediated by macrophages and the apoptosis of macrophages [24–26]. It is

suggested that CSN6, a subunit of the CSN, is cleaved by caspase-8 [27], which is activated by TNF-α during apoptosis [28]. Accumulation of excess free cholesterol may induce apoptosis and secretion of TNF-α to activate caspase-8; subsequently, caspase-8 inhibits the CSN through the cleavage of CSN6. Dysfunction of the CSN could lead to further inflammation and abnormal ABCA1 degradation in advanced plaque.

ABCA1 and ABCG1 have a synergistic role mediating macrophage cholesterol efflux and protecting against macrophage foam cell formation, inflammatory cell infiltration, apoptosis, and atherosclerosis [29]. Inhibition of ABCA1 degradation is one way to suppress plaque formation by exporting free cholesterol from cells via breaking the vicious circle among cholesterol accumulation, apoptosis, and inflammatory responses. Thus, further study to reveal the mechanism of proteasomal degradation of ABCA1, such as the identification of ubiquitin ligase of ABCA1, will be important to develop potential drugs to prevent atherosclerosis.
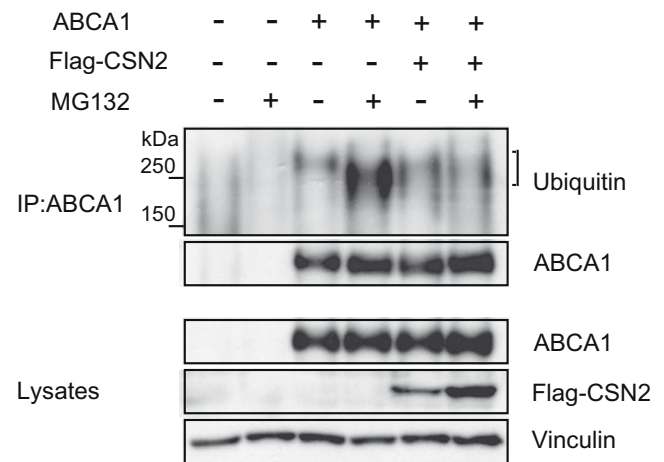

Fig. 4. CSN promotes deubiquitinylation of ABCA1. HEK293 cells were transiently cotransfected with ABCA1 and mock vector or Flag-CSN2. Twenty-four hours after transfection, cells were incubated with or without 50 μM MG132 for 4 h. Cell lysates were immunoprecipitated (IP) with the antibody against ABCA1. Immunocomplex and cell lysates were subjected to immunoblotting using antibodies against ubiquitin, ABCA1, Flag and vinculin. Vinculin was used as a loading control.
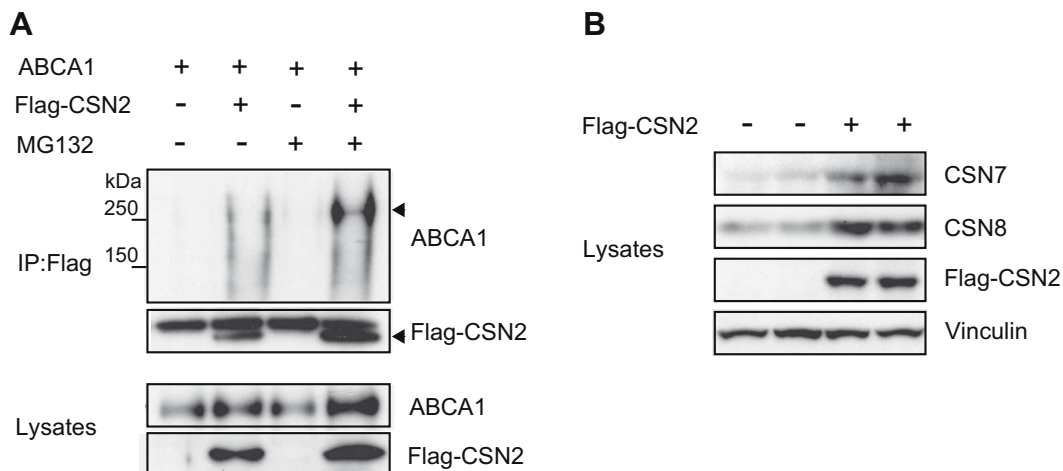

Fig. 3. CSN2 expressed in HEK293 cells associates with ABCA1 and enhances expression of endogenous CSN7 and CSN8. (A) HEK293 cells were transiently cotransfected with ABCA1 and Flag-CSN2. Twenty-four hours after transfection, cells were incubated with or without 50 μM MG132 for 4 h. Cell lysates were immunoprecipitated (IP) with anti-Flag antibody. Immunocomplex and cell lysates were subjected to immunoblotting using antibodies against ABCA1 and Flag. (B) HEK293 cells were transiently transfected with mock vector or Flag-CSN2. Cell lysates were subjected to immunoblotting using antibodies against Flag and endogenous CSN7 and CSN8.

Acknowledgments

References

[1] J.F. Oram, A.M. Vaughan, ATP-binding cassette cholesterol transporters and cardiovascular disease, Circ. Res. 99 (2006) 1031–1043.

[2] S. Yokoyama, Release of cellular cholesterol: molecular mechanism for cholesterol homeostasis in cells and in the body, Biochim. Biophys. Acta 1529 (2000) 231–244.

[3] A.R. Tanaka, S. Abe-Dohmae, T. Ohnishi, R. Aoki, G. Morinaga, K.I. Okuhira, Y. Ikeda, F. Kano, M. Matsuo, N. Kioka, T. Amachi, M. Murata, S. Yokoyama, K. Ueda, Effects of mutations of ABCA1 in the first extracellular domain on subcellular trafficking and ATP binding/hydrolysis, J. Biol. Chem. 278 (2003) 8815–8819.

[4] A. Brooks-Wilson, M. Marcil, S. Clee, L. Zhang, K. Roomp, M. van Dam, L. Yu, C. Brewer, J. Collins, H. Molhuizen, O. Loubser, B. Ouelette, K. Fichter, K. Ashbourne-Excoffon, C. Sensen, S. Scherer, S. Mott, M. Denis, D. Martindale, J. Frohlich, K. Morgan, B. Koop, S. Pimstone, J. Kastelein, M. Hayden, Mutations in ABC1 in Tangier disease and familial high-density lipoprotein deficiency, Nat. Genet. 22 (1999) 336–345.

[5] M. Bodzioch, E. Orso, J. Klucken, T. Langmann, A. Bottcher, W. Diederich, W. Drobnik, S. Barlage, C. Buchler, M. Porsch-Ozcurumez, W. Kaminski, H. Hahmann, K. Oette, G. Rothe, C. Aslanidis, K. Lackner, G. Schmitz, The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease, Nat. Genet. 22 (1999) 347–351.

[6] S. Rust, M. Rosier, H. Funke, J. Real, Z. Amoura, J. Piette, J. Deleuze, H. Brewer, N. Duverger, P. Denefle, G. Assmann, Tangier disease is caused by mutations in the gene encoding ATP-binding cassette transporter 1, Nat. Genet. 22 (1999) 352–355.

[7] R.R. Singaraja, L.R. Brunham, H. Visscher, J.J. Kastelein, M.R. Hayden, Efflux and atherosclerosis: the clinical and biochemical impact of variations in the ABCA1 gene, Arterioscler. Thromb. Vasc. Biol. 23 (2003) 1322–1332.

[8] M. Hozoji, Y. Munehira, Y. Ikeda, M. Makishima, M. Matsuo, N. Kioka, K. Ueda, Direct interaction of nuclear liver X receptor-beta with ABCA1 modulates cholesterol efflux, J. Biol. Chem. 283 (2008) 30057–30063.

[9] Y. Munehira, T. Ohnishi, S. Kawamoto, A. Furuya, K. Shitara, M. Imamura, T. Yokota, S. Takeda, T. Amachi, M. Matsuo, N. Kioka, K. Ueda, Alpha1-syntrophin modulates turnover of ABCA1, J. Biol. Chem. 279 (2004) 15091–15095.

[10] K. Okuhira, M.L. Fitzgerald, D.A. Sarracino, J.J. Manning, S.A. Bell, J.L. Goss, M.W. Freeman, Purification of ATP-binding cassette transporter A1 and associated binding proteins reveals the importance of beta1-syntrophin in cholesterol efflux, J. Biol. Chem. 280 (2005) 39653–39664.

[11] E.B. Neufeld, A.T. Remaley, S.J. Demosky, J.A. Stonik, A.M. Cooney, M. Comly, N.K. Dwyer, M. Zhang, J. Blanchette-Mackie, S. Santamarina-Fojo, H.B. Brewer Jr., Cellular localization and trafficking of the human ABCA1 transporter, J. Biol. Chem. 276 (2001) 27584–27590.

[12] Y. Azuma, M. Takada, H.Y. Shin, N. Kioka, K. Nakayama, K. Ueda, Retroendocytosis pathway of ABCA1/apoA-I contributes to HDL formation, Genes Cells 14 (2009) 191–204.

[13] R. Arakawa, S. Yokoyama, Helical apolipoproteins stabilize ATP-binding cassette transporter A1 by protecting it from thiol protease-mediated degradation, J. Biol. Chem. 277 (2002) 22426–22429.

[14] N. Wang, W. Chen, P. Linsel-Nitschke, L.O. Martinez, B. Agerholm-Larsen, D.L. Silver, A.R. Tall, A PEST sequence in ABCA1 regulates degradation by calpain protease and stabilization of ABCA1 by apoA-I, J. Clin. Invest. 111 (2003) 99–107.

[15] R. Lu, R. Arakawa, C. Ito-Osumi, N. Iwamoto, S. Yokoyama, ApoA-I facilitates ABCA1 recycle/accumulation to cell surface by inhibiting its intracellular degradation and increases HDL generation, Arterioscler. Thromb. Vasc. Biol. 28 (2008) 1820–1824.

[16] B. Feng, I. Tabas, ABCA1-mediated cholesterol efflux is defective in free cholesterol-loaded macrophages. Mechanism involves enhanced ABCA1 degradation in a process requiring full NPC1 activity, J. Biol. Chem. 277 (2002) 43271–43280.

[17] K. Schweitzer, P.M. Bozko, W. Dubiel, M. Naumann, CSN controls NF-kappaB by deubiquitinylation of IkappaBalpha, EMBO J. 26 (2007) 1532–1541.

[18] G.A. Cope, G.S. Suh, L. Aravind, S.E. Schwarz, S.L. Zipursky, E.V. Koonin, R.J. Deshaies, Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1, Science 298 (2002) 608–611.

[19] T. Tsuge, M. Matsui, N. Wei, The subunit 1 of the COP9 signalosome suppresses gene expression through its N-terminal domain and incorporates into the complex through the PCI domain, J. Mol. Biol. 305 (2001) 1–9.

[20] M. Naumann, D. Bech-Otschir, X. Huang, K. Ferrell, W. Dubiel, COP9 signalosome-directed c-Jun activation/stabilization is independent of JNK, J. Biol. Chem. 274 (1999) 35297–35300.

[21] S. Denti, M.E. Fernandez-Sanchez, L. Rogge, E. Bianchi, The COP9 signalosome regulates Skp2 levels and proliferation of human cells, J. Biol. Chem. 281 (2006) 32188–32196.

[22] E. Bianchi, S. Denti, A. Granata, G. Bossi, J. Geginat, A. Villa, L. Rogge, R. Pardi, Integrin LFA-1 interacts with the transcriptional co-activator JAB1 to modulate AP-1 activity, Nature 404 (2000) 617–621.

[23] J. Yun, A. Tomida, T. Andoh, T. Tsuruo, Interaction between glucose-regulated destruction domain of DNA topoisomerase IIalpha and MPN domain of Jab1/CSN5, J. Biol. Chem. 279 (2004) 31296–31303.

[24] T. Devries-Seimon, Y. Li, P.M. Yao, E. Stone, Y. Wang, R.J. Davis, R. Flavell, I. Tabas, Cholesterol-induced macrophage apoptosis requires ER stress pathways and engagement of the type A scavenger receptor, J. Cell Biol. 171 (2005) 61–73.

[25] Y. Li, R.F. Schwabe, T. DeVries-Seimon, P.M. Yao, M.C. Gerbod-Giannone, A.R. Tall, R.J. Davis, R. Flavell, D.A. Brenner, I. Tabas, Free cholesterol-loaded macrophages are an abundant source of tumor necrosis factor-alpha and interleukin-6: model of NF-kappaB- and map kinase-dependent inflammation in advanced atherosclerosis, J. Biol. Chem. 280 (2005) 21763–21772.

[26] F.R. Maxfield, I. Tabas, Role of cholesterol and lipid organization in disease, Nature 438 (2005) 612–621.

[27] J. da Silva Correia, Y. Miranda, N. Leonard, R.J. Ulevitch, The subunit CSN6 of the COP9 signalosome is cleaved during apoptosis, J. Biol. Chem. 282 (2007) 12557–12565.

[28] L. Wang, F. Du, X. Wang, TNF-alpha induces two distinct caspase-8 activation pathways, Cell 133 (2008) 693–703.

[29] L. Yvan-Charvet, M. Ranalletta, N. Wang, S. Han, N. Terasaka, R. Li, C. Welch, A.R. Tall, Combined deficiency of ABCA1 and ABCG1 promotes foam cell accumulation and accelerates atherosclerosis in mice, J. Clin. Invest. 117 (2007) 3900–3908.