

Supporting Information

Mechanisms that minimize retinal impact of apolipoprotein E absence

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Supporting Text 1. Gene and protein symbol abbreviations used in the present work. Gene symbols are italicized and begin with an uppercase letter; protein symbols have all letters in uppercase.

Gene Abbreviations

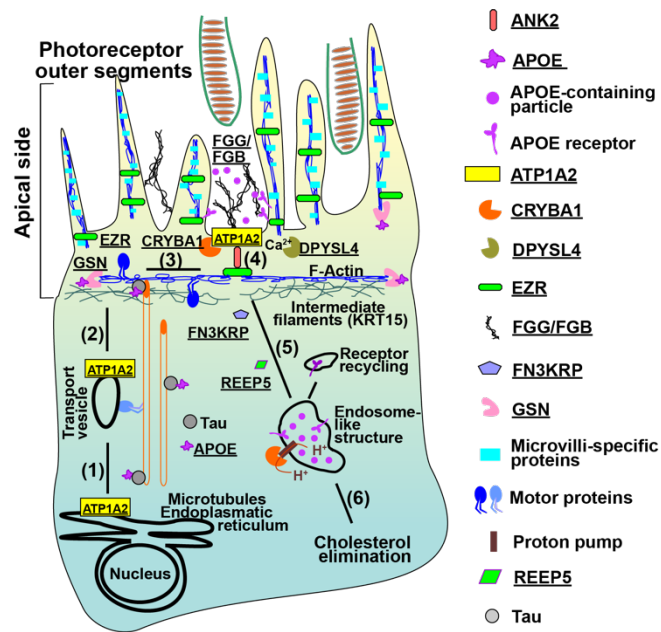
Apoa2, *Apob*, *Apoc3*, *Apod*, *ApoE*, and *ApoF* (apolipoproteins A2, B, C3, D, E, and F, respectively); *Ccl2* (C-C motif chemokine 2), *Cox-2* (prostaglandin G/H synthase 2), *Idol* (E3 ubiquitin-protein ligase MYLIP), *Il-6* (interleukin-6), *Il-1 β* (interleukin-1 β), *iNos* (nitric oxide synthase, inducible), *Ldlr*, receptor for low density lipoprotein; *Lpl*, lipoprotein lipase; *Lxra* (oxysterols receptor LXR-alpha), *Lxr β* (oxysterols receptor LXR-beta), *Tnfa* (tumor necrosis factor).

Protein Abbreviations

ACTB (actin β), ALDOA (aldolase A), ANK2 (ankyrin 2), apolipoprotein A1 (APOA1), apolipoprotein A4 (APOA4), apolipoprotein E (APOE), apolipoprotein J (APOJ), ATP1A2 (ATPase Na⁺/K⁺ transporting subunit alpha 2), ATP5B (ATP synthase F1 subunit beta), CER (ceruloplasmin protein), CKB (creatine kinase B), CRYBA1 (crystallin beta A1), CRYAA2 (crystallin alpha A2), CRYBB1 (crystallin beta B1), CRYBB2 (crystallin beta B2), DHCR7 (7-dehydrocholesterol reductase), DPYSL4 (dihydropyriminidase like 4), ENO1 (enolase), EZR (ezrin), FGB (fibrinogen beta chain), FGG (fibrinogen gamma chain), FN3KRP (fructosamine 3 kinase related protein), FPN (ferroportin); FTH1 (ferritin heavy chain 1), GAPDH (glyceraldehyde 3 phosphate dehydrogenase), GNAT1 (G protein subunit alpha transducin 1), GNB1 (G protein subunit beta 1), GNGT1 (G protein subunit gamma transducin 1), GSN (gelsolin), HDL (high density lipoprotein), HIST1H1C (histone cluster 1 H1 family member C), HIST1H1E (histone cluster 1 H1 family member E), HIST1H2AF (histone cluster 1 H2af), HIST1H2BB (histone cluster 1 H2B family member B), HIST1H3A ((histone cluster 1 H3 family member A), HIST1H4K (histone cluster 1 H4 family member K), HMGN5 (high mobility group nucleosome binding domain 5), IDL (intermediate density lipoprotein), KRT15 (keratin 15), LDHA (lactose dehydrogenase A), LDL (low density lipoprotein), LRP1 (low density lipoprotein receptor-related protein 1), LRPAP1 (low density lipoprotein-related protein-associated), LZIC (leucine zipper and ICAT homologous domain-containing protein), MESDC2 (mesoderm development LRP chaperone), OSBPL1A (oxysterol binding protein like 1A), OSBPL2 (oxysterol binding protein like 2), OSBP1 (oxysterol binding protein 1), OSBP2 (oxysterol binding protein 2), OSBPL8 (oxysterol binding protein like 8), PGAM1 (phosphoglycerate mutase 1), PGK1 (phosphoglycerate kinase 1), PKM (pyruvate kinase M1/2), PON1 (paraoxonase 1), PON2 (paraoxonase 2), PTMS (parathymosin), REEP5 (receptor accessory protein 5), RHO (rhodopsin), SAG (S-antigen visual arrestin), SCARB2 (scavenger receptor class B member 2), SCG2 (secretogranin II), SORT1 (sortilin 1), TF (transferrin), TFR (transferrin receptor), TUBA1A (tubulin alpha 1a), VLDL (very low density lipoprotein), WDFY1 (WD repeat and FYVE domain containing 1), and ZC3H4 (zinc finger CCCH domain-containing protein 4).

Supporting Table S1. Primers for Quantitative Real-Time PCR.

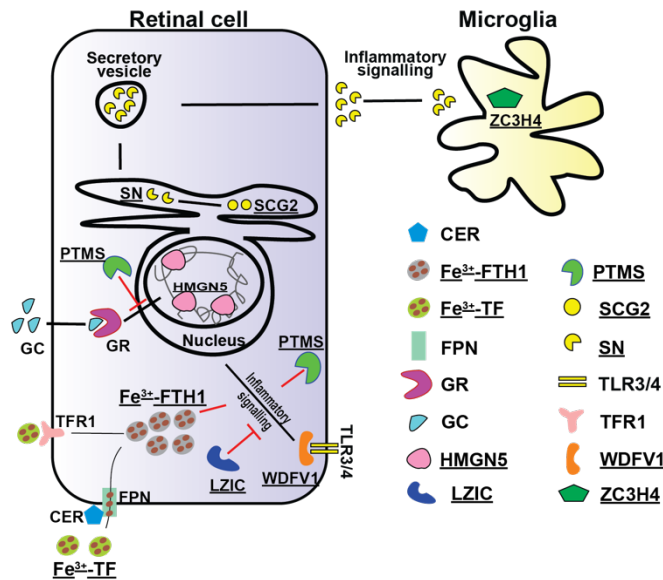
Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
<i>Apoa2</i>	CCCGTGGTCATCGTCTCAG	GGACAGGGCACCATTGAAGG
<i>Apob</i>	TTTGCCCTCAACCTACCAAC	TGCGATCTTGTTGGCTACTG
<i>Apoc3</i>	G TTCACCGGCTTCTGGGATT	CAACACAGAAGTCTCACGACTCAA
<i>Apod</i>	TGAAGCCAAACAGAGCAACGT	GGCATCAACGGGAAGAAGT
<i>Apof</i>	CATCCACAGGACACAACAGG	TCTCAATGGCCGGACTGT
<i>Ccl2</i>	TACTGAAGCCAGCTCTCTCTTC	GTGAACAGCAGGCCAGAA
<i>Cox-2</i>	CACCTCAAGAACATCCAGAGCTT	CTCGCGACCATTCTTGAGTGT
<i>Idol</i>	GGAGCATGTCCAGCACGTCTA	GTGCAGGACGCATCAGATGA
<i>Il-1β</i>	GGTCAAAGGTTTGGAAGCAG	TGTGAAATGCCACCTTTTGA
<i>Il-6</i>	AGTTGCCTTCTTGGGACTGA	TCCACGATTTCCCAGAGAAC
<i>iNos</i>	GCCACCAACAATGGCAACA	CGTACCGGATGAGCTGTGAA
<i>Ldlr</i>	ACCTGCCGACCTGATGAATTC	GCAGTCATGTTACGGTCACA
<i>Lpl</i>	TTTATCCCAATGGAGGCACTTT	CAATCACACGGATGGCTTCTC
<i>Lxrα</i>	AGCGTCCATTCAGAGCAAGTG	CACTCGTGGACATCCCAGATCT
<i>Lxrβ</i>	ACTCGGAGCAGGTCTTTGCAT	CCTACTCGTGCACATCCCAGAT
<i>Tnfβ</i>	GGTCTGGGCCATAGAAGTGA	CAGCCTCTTCTCATTCTGC



Supporting Figure S1. Putative links between APOE and retinal proteins with decreased expression in *ApoE*^{-/-} mice.

Events are numbered (in parenthesis), and downregulated proteins are underlined>. ATP1A2 is central in all these links, and the major events are hypothesized to take place in the RPE. (1) ATP1A2 is synthesized in the endoplasmic reticulum and (2) transported to the apical membrane by vesicles, whose traffic is mediated by motor proteins along microtubules (1,2). Tau, the APOE-interacting protein, is required for microtubule-dependent traffic and mediates interaction between microtubules and actin filaments (3,4). (3) After reaching the apical side, another motor proteins carry transport vesicles along actin filaments (1) followed by

(4) vesicle incorporation into the apical plasma membranes by exocytosis (5). GSN, another APOE-interacting protein, regulates actin-dependent traffic to plasma membranes, in part by capping actin filaments (6-8). Once in the plasma membrane, ATP1A2 may form a complex with EZR bridged by ANK2 and be connected to actin cytoskeleton *via* EZR (2,9-14). The precise location of this complex may require the interaction of F-actin with intermediate filaments including KRT15 (15,16). In addition, ATP1A2 could interact directly with CRYBA1 and FGG/FGB and perhaps also affect the activity (*via* the intracellular Ca²⁺ levels) of DPYSL4 (17-21). Furthermore, the complex of ATP1A2 with ANK2 (EZR), CRYBA1, and FGG/FGB is suggested to participate in the uptake of extracellular fluid phase (not shown), which may include APOE-containing particles (2,21-23). (5) FN3KRP and REEP5 may be required for subsequent formation of endosome-like structures (24-26), which when are formed, (6) could eliminate the up-taken cholesterol *via* different vesicular and non-vesicular pathways. Notably, CRYBA1 may regulate the acidification of endosomes (27), an important step for cholesterol efflux from endolysosomes. In addition to cytoskeleton inside the RPE, there is a cytoskeleton inside the RPE microvilli, which is composed of actin filaments interconnected by EZR and other proteins (28). Similar to intracellular actin filaments, this cytoskeleton is capped by GSN, which interacts with APOE. The detected changes in protein abundance in *ApoE*^{-/-} mice (Tables 2,3) suggest that a lack of APOE may decrease the RPE cytoskeleton stability both inside the cell and microvilli and thereby impair the cytoskeleton-mediated vesicular delivery of ATP1A2 to the apical RPE aspect as well as the microvilli structure. As a result, there may be a decrease in ATP1A2 abundance at the apical RPE side and a corresponding decrease in the expression of proteins, which interact with this enzyme (ANK2, EZR, CRYBA1, FGG/FGB, DPYSL4, GSN, FN3KRP, REEP5), directly or indirectly. Also, effect on the RPE microvilli structure may affect the photoreceptor phagocytosis as well as APOE-receptors (LDLR, VLDLR, LRP1) on the apical RPE aspect (29,30) and diminish the RPE uptake of retinal cholesterol.



Supporting Figure S2. Putative links between APOE and retinal proteins with increased abundance in *Apoe*^{-/-} mice. Arrows indicate activation, blunt-ends denote repression or inhibition. The upregulated proteins are underlined. The levels of FTH1 (ferritin heavy chain, the main intracellular iron storage protein) were increased in the *Apoe*^{-/-} retina (Table 2), indicating an increase in retinal iron levels (31,32). Yet, the levels of proteins involved in cellular iron import (transferrin, TF, and transferrin receptor, TFR) and export (ferroportin, FPN, and ceruloplasmin, CER) were unchanged in the *Apoe*^{-/-} retina, thus suggesting that the RPE phagocytosis is

somewhat impaired or slow (possibly due to decreased levels of ATP1A2, ANK2, and EZR, Suppl. Fig. S1). If so, changes in the photoreceptor phagocytosis, a process playing multiple roles including iron export from the neural retina, can affect iron homeostasis in the neural retina and result in iron accumulation in retinal cells. The latter may lead to upregulation in retinal cells of both pro-inflammatory PTMS (parathyrosin), WDFV1 (WD repeat and FYVE domain-containing protein 1), SCG2 (secretogranin-2), and ZC3H4 (zinc finger CCCH domain-containing protein 4) as well as anti-inflammatory LZIC (leucine zipper and ICAT homologous domain-containing protein) and HMGNS (high mobility group nucleosome-binding domain-containing protein 5). Increased PTMS (immune-related polypeptide) levels may increase the inhibition of glucocorticoid-receptor binding to nuclei and in addition antagonism of prothymosin (not shown), which contributes to protective toll-like receptor (TLR) 4 signaling (33-35). Increased abundance of WDFV1 (an adaptor protein) could enhance activation of NF- κ B (not shown, controls the inflammatory gene expression) *via* TLR4 as well as TLR3 (36). Finally, increased expression of SCG2 (a neurosecretory protein) and subsequent formation of SN (secretoneurin) from SCG2 can lead to microglia attraction/activation (37-39) as indicated by increased abundance of ZC3H4 (a newly identified protein similar to monocyte chemotactic protein 1-induced protein 1) (40). SCG2 can also exert neuroprotective properties (37,41) and suppress oxidative stress (42). Similarly, increased levels of HMGNS (a nucleosome binding protein and chromosome guardian (43,44)) and LZIC (a putative signal transduction protein required for neuronal survival) (45) could be compensatory and reduce the effects of pro-inflammatory PTMS, WDFV1, SCG2, and ZC3H4.

Supporting Information References

1. Williams, D. S., and Lopes, V. S. (2011) The many different cellular functions of MYO7A in the retina. *Biochem Soc Trans* **39**, 1207-1210
2. Lobato-Alvarez, J. A., Roldan, M. L., Lopez-Murillo, T. D., Gonzalez-Ramirez, R., Bonilla-Delgado, J., and Shoshani, L. (2016) The Apical Localization of Na(+), K(+)-ATPase in Cultured Human Retinal Pigment Epithelial Cells Depends on Expression of the beta2 Subunit. *Frontiers in physiology* **7**, 450
3. Mohan, R., and John, A. (2015) Microtubule-associated proteins as direct crosslinkers of actin filaments and microtubules. *IUBMB Life* **67**, 395-403
4. Fleming, L. M., Weisgraber, K. H., Strittmatter, W. J., Troncoso, J. C., and Johnson, G. V. (1996) Differential binding of apolipoprotein E isoforms to tau and other cytoskeletal proteins. *Exp Neurol* **138**, 252-260
5. Lehmann, G. L., Benedicto, I., Philp, N. J., and Rodriguez-Boulan, E. (2014) Plasma membrane protein polarity and trafficking in RPE cells: past, present and future. *Exp Eye Res* **126**, 5-15
6. Baumann, M. H., Kallijarvi, J., Lankinen, H., Soto, C., and Haltia, M. (2000) Apolipoprotein E includes a binding site which is recognized by several amyloidogenic polypeptides. *Biochem J* **349**, 77-84
7. Hartwig, J. H., Brown, D., Ausiello, D. A., Stossel, T. P., and Orci, L. (1990) Polarization of gelsolin and actin binding protein in kidney epithelial cells. *J Histochem Cytochem* **38**, 1145-1153
8. Olt, J., Mburu, P., Johnson, S. L., Parker, A., Kuhn, S., Bowl, M., Marcotti, W., and Brown, S. D. (2014) The actin-binding proteins eps8 and gelsolin have complementary roles in regulating the growth and stability of mechanosensory hair bundles of mammalian cochlear outer hair cells. *PLoS One* **9**, e87331
9. Burke, J. M., Cao, F., and Irving, P. E. (2000) High levels of E-/P-cadherin: correlation with decreased apical polarity of Na/K ATPase in bovine RPE cells in situ. *Invest Ophthalmol Vis Sci* **41**, 1945-1952
10. Bensaoula, T., and Ottlecz, A. (2001) Biochemical and ultrastructural studies in the neural retina and retinal pigment epithelium of STZ-diabetic rats: effect of captopril. *J Ocul Pharmacol Ther* **17**, 573-586
11. Kizhatil, K., Sandhu, N. K., Peachey, N. S., and Bennett, V. (2009) Ankyrin-B is required for coordinated expression of beta-2-spectrin, the Na/K-ATPase and the Na/Ca exchanger in the inner segment of rod photoreceptors. *Exp Eye Res* **88**, 57-64
12. Bonilha, V. L., Rayborn, M. E., Saotome, I., McClatchey, A. I., and Hollyfield, J. G. (2006) Microvilli defects in retinas of ezrin knockout mice. *Exp Eye Res* **82**, 720-729
13. Kivela, T., Jaaskelainen, J., Vaheri, A., and Carpen, O. (2000) Ezrin, a membrane-organizing protein, as a polarization marker of the retinal pigment epithelium in vertebrates. *Cell Tissue Res* **301**, 217-223
14. Luna, G., Lewis, G. P., Linberg, K. A., Chang, B., Hu, Q., Munson, P. J., Maminishkis, A., Miller, S. S., and Fisher, S. K. (2016) Anatomical and Gene Expression Changes in the Retinal Pigmented Epithelium Atrophy 1 (rpeal) Mouse: A Potential Model of Serous Retinal Detachment. *Invest Ophthalmol Vis Sci* **57**, 4641-4654
15. Coch, R. A., and Leube, R. E. (2016) Intermediate Filaments and Polarization in the Intestinal Epithelium. *Cells* **5**

16. Wald, F. A., Oriolo, A. S., Casanova, M. L., and Salas, P. J. (2005) Intermediate filaments interact with dormant ezrin in intestinal epithelial cells. *Mol Biol Cell* **16**, 4096-4107
17. Valapala, M., Sergeev, Y., Wawrousek, E., Hose, S., Zigler, J. S., Jr., and Sinha, D. (2016) Modulation of V-ATPase by betaA3/A1-Crystallin in Retinal Pigment Epithelial Cells. *Adv Exp Med Biol* **854**, 779-784
18. Derham, B. K., Ellory, J. C., Bron, A. J., and Harding, J. J. (2003) The molecular chaperone alpha-crystallin incorporated into red cell ghosts protects membrane Na/K-ATPase against glycation and oxidative stress. *Eur J Biochem* **270**, 2605-2611
19. Thanos, S., Bohm, M. R., Meyer zu Horste, M., Prokosch-Willing, V., Hennig, M., Bauer, D., and Heiligenhaus, A. (2014) Role of crystallins in ocular neuroprotection and axonal regeneration. *Prog Retin Eye Res* **42**, 145-161
20. Quach, T. T., Wilson, S. M., Rogemond, V., Chounlamountri, N., Kolattukudy, P. E., Martinez, S., Khanna, M., Belin, M. F., Khanna, R., Honnorat, J., and Duchemin, A. M. (2013) Mapping CRMP3 domains involved in dendrite morphogenesis and voltage-gated calcium channel regulation. *J Cell Sci* **126**, 4262-4273
21. Muradashvili, N., Khundmiri, S. J., Tyagi, R., Gartung, A., Dean, W. L., Lee, M. J., and Lominadze, D. (2014) Sphingolipids affect fibrinogen-induced caveolar transcytosis and cerebrovascular permeability. *American journal of physiology. Cell physiology* **307**, C169-179
22. Gallicchio, M. A., and Bach, L. A. (2013) Uptake of advanced glycation end products by proximal tubule epithelial cells via macropinocytosis. *Biochim Biophys Acta* **1833**, 2922-2932
23. Smith, P. M., Cowan, A., and White, B. A. (2004) The low-density lipoprotein receptor is regulated by estrogen and forms a functional complex with the estrogen-regulated protein ezrin in pituitary GH3 somatotropes. *Endocrinology* **145**, 3075-3083
24. Pelkmans, L., Fava, E., Grabner, H., Hannus, M., Habermann, B., Krausz, E., and Zerial, M. (2005) Genome-wide analysis of human kinases in clathrin- and caveolae/raft-mediated endocytosis. *Nature* **436**, 78-86
25. Park, C. R., You, D. J., Park, S., Mander, S., Jang, D. E., Yeom, S. C., Oh, S. H., Ahn, C., Lee, S. H., Seong, J. Y., and Hwang, J. I. (2016) The accessory proteins REEP5 and REEP6 refine CXCR1-mediated cellular responses and lung cancer progression. *Sci Rep* **6**, 39041
26. Veleri, S., Nellisery, J., Mishra, B., Manjunath, S. H., Brooks, M. J., Dong, L., Nagashima, K., Qian, H., Gao, C., Sergeev, Y. V., Huang, X. F., Qu, J., Lu, F., Cideciyan, A. V., Li, T., Jin, Z. B., Fariss, R. N., Ratnapriya, R., Jacobson, S. G., and Swaroop, A. (2017) REEP6 mediates trafficking of a subset of Clathrin-coated vesicles and is critical for rod photoreceptor function and survival. *Hum Mol Genet* **26**, 2218-2230
27. Valapala, M., Wilson, C., Hose, S., Bhutto, I. A., Grebe, R., Dong, A., Greenbaum, S., Gu, L., Sengupta, S., Cano, M., Hackett, S., Xu, G., Luty, G. A., Dong, L., Sergeev, Y., Handa, J. T., Campochiaro, P., Wawrousek, E., Zigler, J. S., Jr., and Sinha, D. (2014) Lysosomal-mediated waste clearance in retinal pigment epithelial cells is regulated by CRYBA1/betaA3/A1-crystallin via V-ATPase-MTORC1 signaling. *Autophagy* **10**, 480-496
28. Bonilha, V. L., Rayborn, M. E., Bhattacharya, S. K., Gu, X., Crabb, J. S., Crabb, J. W., and Hollyfield, J. G. (2006) The retinal pigment epithelium apical microvilli and retinal function. *Adv Exp Med Biol* **572**, 519-524
29. Fliesler, S. J., and Bretillon, L. (2010) The ins and outs of cholesterol in the vertebrate retina. *J Lipid Res* **51**, 3399-3413

30. Hollborn, M., Birkenmeier, G., Saalbach, A., Iandiev, I., Reichenbach, A., Wiedemann, P., and Kohen, L. (2004) Expression of LRP1 in retinal pigment epithelial cells and its regulation by growth factors. *Invest Ophthalmol Vis Sci* **45**, 2033-2038
31. Song, D., and Dunaief, J. L. (2013) Retinal iron homeostasis in health and disease. *Front Aging Neurosci* **5**, 24
32. Arosio, P., Elia, L., and Poli, M. (2017) Ferritin, cellular iron storage and regulation. *IUBMB life* **69**, 414-422
33. Halder, S. K., Matsunaga, H., Ishii, K. J., and Ueda, H. (2015) Prothymosin-alpha preconditioning activates TLR4-TRIF signaling to induce protection of ischemic retina. *J Neurochem* **135**, 1161-1177
34. Hannappel, E., and Huff, T. (2003) The thymosins. Prothymosin alpha, parathymosin, and beta-thymosins: structure and function. *Vitam Horm* **66**, 257-296
35. Okamoto, K., and Isohashi, F. (2005) Macromolecular translocation inhibitor II (Zn(2+)-binding protein, parathymosin) interacts with the glucocorticoid receptor and enhances transcription in vivo. *J Biol Chem* **280**, 36986-36993
36. Hu, Y. H., Zhang, Y., Jiang, L. Q., Wang, S., Lei, C. Q., Sun, M. S., Shu, H. B., and Liu, Y. (2015) WDFY1 mediates TLR3/4 signaling by recruiting TRIF. *EMBO reports* **16**, 447-455
37. Mattsson, N., Ruetschi, U., Podust, V. N., Stridsberg, M., Li, S., Andersen, O., Haghghi, S., Blennow, K., and Zetterberg, H. (2007) Cerebrospinal fluid concentrations of peptides derived from chromogranin B and secretogranin II are decreased in multiple sclerosis. *J Neurochem* **103**, 1932-1939
38. Shooshtarizadeh, P., Zhang, D., Chich, J. F., Gasnier, C., Schneider, F., Haikel, Y., Aunis, D., and Metz-Boutigue, M. H. (2010) The antimicrobial peptides derived from chromogranin/secretogranin family, new actors of innate immunity. *Regul Pept* **165**, 102-110
39. Schratzberger, P., Woll, E., Reinisch, N., Kahler, C. M., and Wiedemann, C. J. (1996) Secretoneurin-induced in vitro chemotaxis of human monocytes is inhibited by pertussis toxin and an inhibitor of protein kinase C. *Neurosci Lett* **214**, 208-210
40. Yang, X., Wang, J., Zhou, Z., Jiang, R., Huang, J., Chen, L., Cao, Z., Chu, H., Han, B., Cheng, Y., and Chao, J. (2018) Silica-induced initiation of circular ZC3H4 RNA/ZC3H4 pathway promotes the pulmonary macrophage activation. *FASEB J*, fj201701118R
41. Posod, A., Wechselberger, K., Stanika, R. I., Obermair, G. J., Wegleiter, K., Huber, E., Urbanek, M., Kiechl-Kohlendorfer, U., and Griesmaier, E. (2017) Administration of secretoneurin is protective in hypoxic-ischemic neonatal brain injury predominantly in the hypoxic-only hemisphere. *Neuroscience* **352**, 88-96
42. Chen, H. L., Liu, Y., Jiang, W., Wang, X. X., Yuan, G. L., Zhao, Y. L., and Yu, C. (2018) Secretoneurin suppresses cardiac hypertrophy through suppression of oxidant stress. *Eur J Pharmacol* **822**, 13-24
43. Shi, Z., Tang, R., Wu, D., and Sun, X. (2016) Research advances in HMGN5 and cancer. *Tumour Biol* **37**, 1531-1539
44. Moretti, F., Rolando, C., Winker, M., Ivanek, R., Rodriguez, J., Von Kriegsheim, A., Taylor, V., Bustin, M., and Pertz, O. (2015) Growth Cone Localization of the mRNA Encoding the Chromatin Regulator HMGN5 Modulates Neurite Outgrowth. *Molecular and cellular biology* **35**, 2035-2050
45. Clements, W. K., and Kimelman, D. (2005) LZIC regulates neuronal survival during zebrafish development. *Dev Biol* **283**, 322-334