Supplementary data accompanying the manuscript

"Biochemical and pharmacological characterization of human α/β-hydrolase domain containing 6 (ABHD6) and 12 (ABHD12)"

by

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Supplementary Figure 1. Substrate profiles of hABHD6 and hABHD12 from four separate transfections.

Supplementary Figure 2. Relative activity of hABHD6 and hABHD12 in HEK293 cell lysates and membrane preparations.

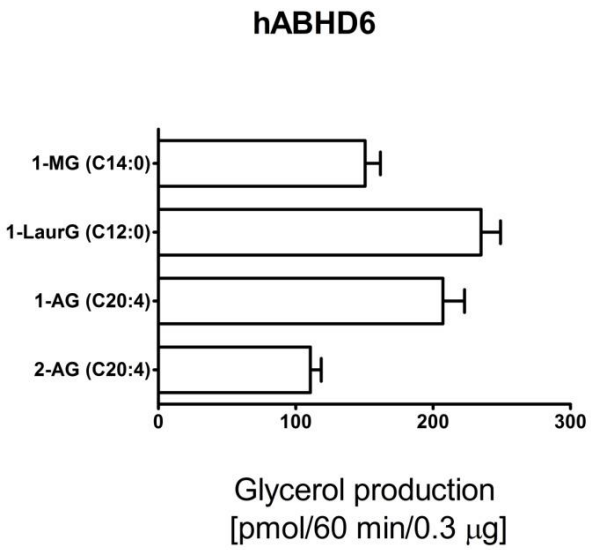
Supplementary Figure 3. pH dependence of hABHD6 and hABHD12 activities.

Supplementary Figure 4. Primary structure comparisons between human, mouse, and rat orthologs of ABHD6 and ABHD12.

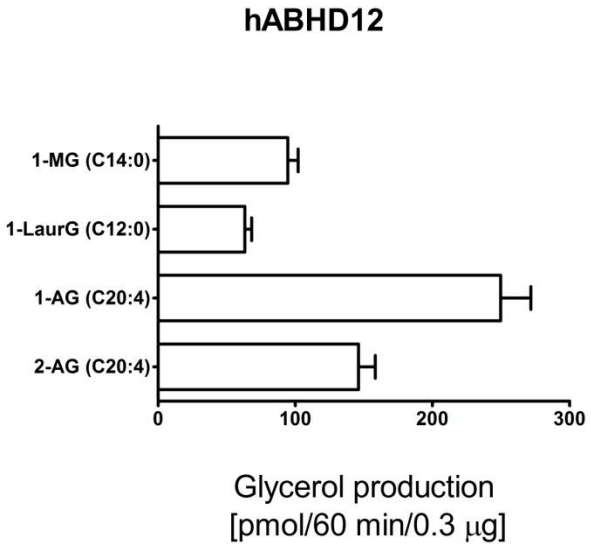
Supplementary Figure 5. Kinetic monitoring of glycerol output from 1(3)-AG and 2-AG by the endocannabinoid hydrolases hABHD6, hABHD12 and hMAGL.

Supplementary Table 1. hABHD6 and hABHD12 possess no detectable fatty acid amide hydrolase (FAAH) activity.

a)

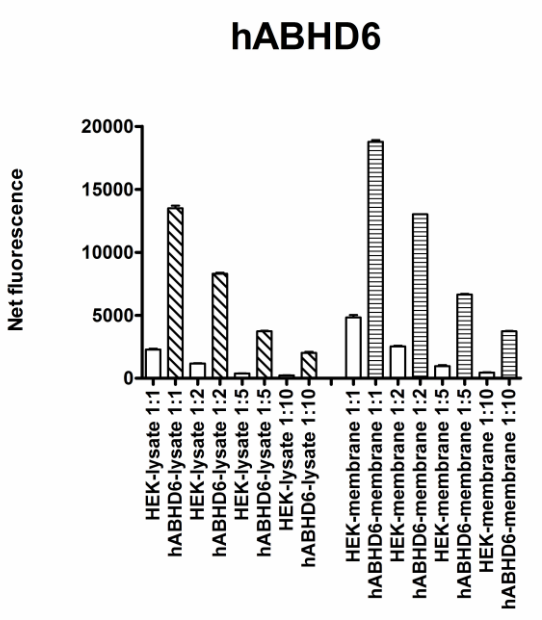


b)

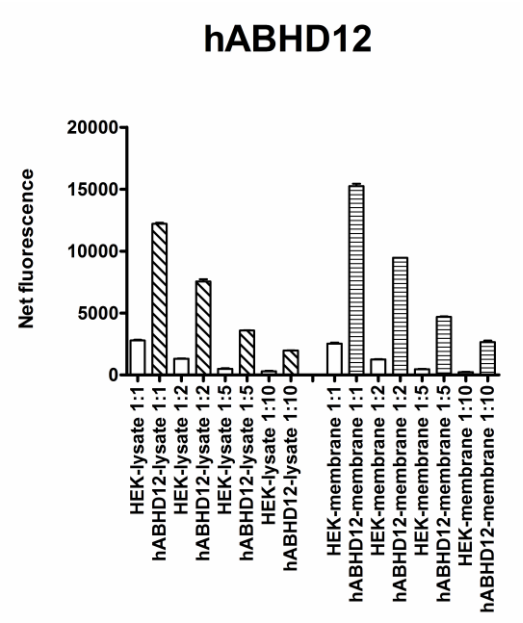


Supplementary Figure 1. Substrate profiles of hABHD6 (a) and hABHD12 (b) as determined from four separate batches of HEK cell lysates (0.3 μg/well) after transient transfections of the two hydrolases. Glycerol was determined following 60 min incubation with the indicated substrates (final substrate concentration 25 μM) and glycerol production determined at time-point 60 min. Data are mean + SEM from lysates from four different transfections.

a)

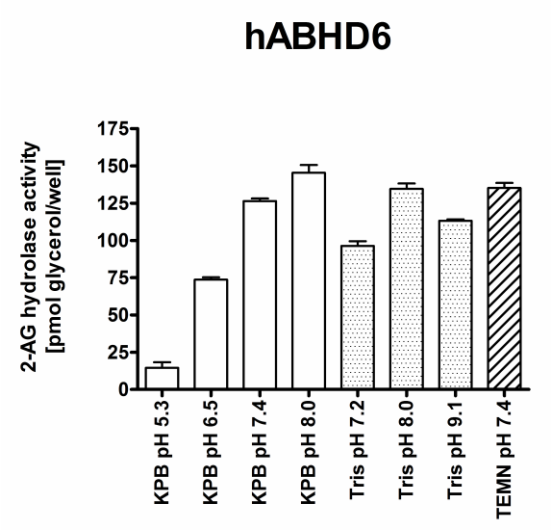


b)

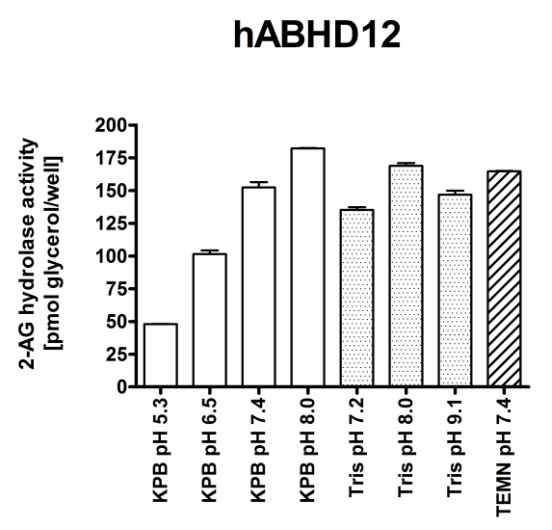


Supplementary Figure 2. Relative activity of hABHD6 (a) and hABHD12 (b) in cellular lysates and membrane preparations. Lysates and membranes were prepared from HEK293 cells transiently overexpressing hABHD6 or hABHD12 as described in "Experimental procedures". Enzymatic activity was determined with increasing protein concentration (0.5-5 µg/well) using 2-AG as the substrate (12.5 µM final concentration), as detailed in Fig. 1b. Dilution 1:1 corresponds to 5.0 µg protein/well. For comparative purposes, cellular background activity is shown at each protein concentration. Data are mean + SD of duplicate wells from one experiment and are expressed as net fluorescence at time-point 60 min with the assay blank values subtracted.

a)



b)

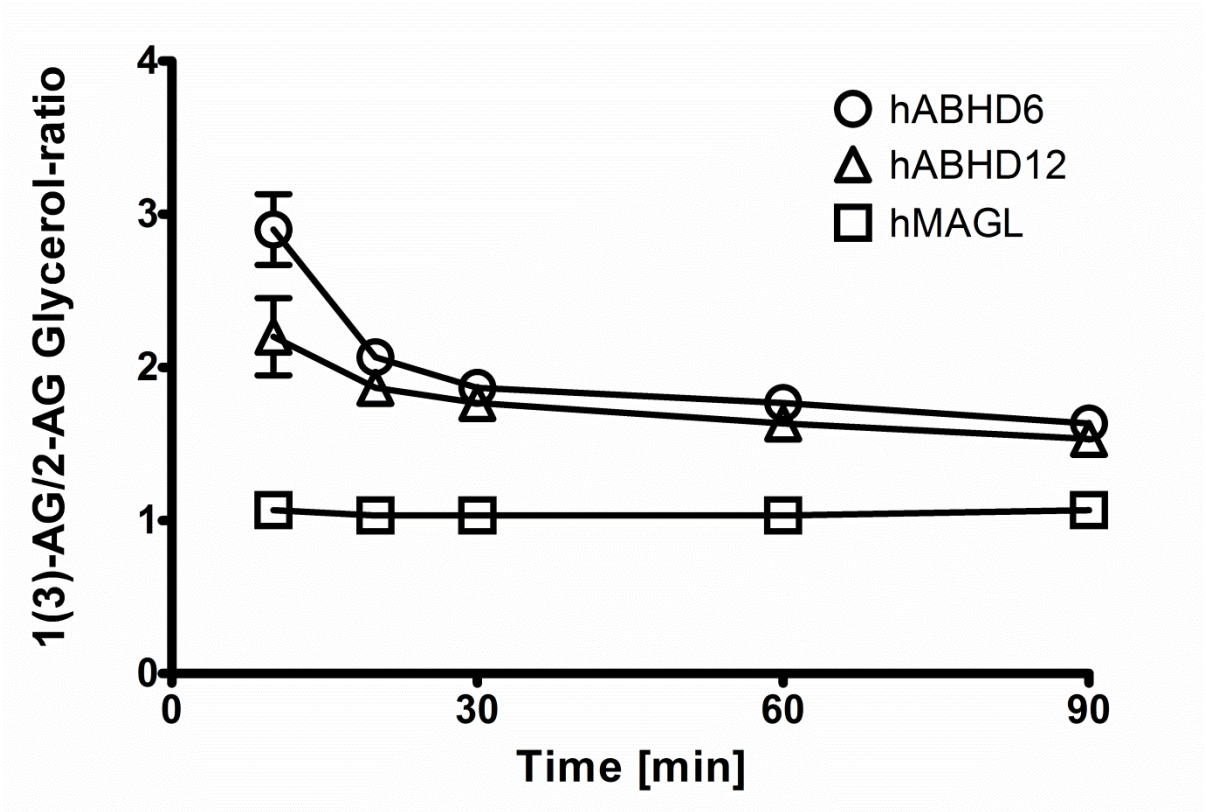


Supplementary Figure 3. pH dependence of hABHD6 (a) and hABHD12 (b) activities. Lysates (0.3 µg/well) of HEK cells transiently overexpressing the two hydrolases were incubated at the indicated pH for 30 min with the substrate 2-AG (25 µM final concentration). The incubations additionally contained 0.1 % (v/v) ethanol, 0.1 % (v/v) DMSO and 0.5 % (w/v) BSA. After this, the pH of the assay system was brought to the neutral range by the addition of Glycerol Assay Mix (Fig. 1b) containing additionally 100 mM Tris-HCl, pH 7.4 and MAFP (10^{-5} M) to quench hydrolase activity. Assay blanks and glycerol standards were included for each pH condition. Glycerol content was determined at time-point 60 min. The buffer systems were as follows: 10 mM K-phosphate buffer (KPB) covering the pH range 5.3 - 8.0 and 10 mM Tris-HCl covering the pH range 7.2 - 9.1. For comparative purposes, hydrolase activity in the routine assay buffer (TEMN, pH 7.4) is also shown. Data are mean + SD of duplicate wells from a representative experiment that was repeated once with similar outcome.

ABHD6

ABHD12

Supplementary Figure 4. Primary structure comparisons between human (h), mouse (m), and rat (r) orthologs of ABHD6 and ABHD12. Sequences were aligned manually based on the frame provided by conserved cysteine residues (C). The lipase motif (**GXSXG/A**) and the predicted residues of the catalytic triad (SDH) are highlighted against red background. Gray shading highlights differences between the species. TM, putative transmembrane domain. The sequence accession numbers were for ABHD6: NP_065727.4 (h), NP_079617 (m), NP_001007681 (r); and for ABHD12: CAI13763.1 (h), CAM18377.1 (m), NP_001019485.1 (r).



Supplementary Figure 5. Time-dependency of glycerol output from the hydrolysis of 1(3)- and 2-isomers of MAG(C20:4) by the three human endocannabinoid hydrolases. Lysates (0.3 µg/well) of HEK293 cells overexpressing the MAG lipases were incubated together with 1(3)-AG or 2-AG [25 µM final concentration, added from 10 mM stock solutions in ethanol into the glycerol assay mix containing 0.5 % (w/v) BSA and 1 % (v/v ethanol). Glycerol production was monitored kinetically for 90 min. Note that in contrast to hMAGL which hydrolyzes both isomers at identical rates, hABHD6 and hABHD12 clearly prefer the 1(3)-isomer. This is particularly evident at the early time-points suggesting that nonenzymatic isomerization of 2-AG to 1(3)-AG is likely needed prior to enzymatic hydrolysis by hABHD6 or hABHD12. Data are mean ± SEM from 3 independent experiments.

Supplementary Table 1. Lack of fatty acid amide hydrolase (FAAH) activity of lysates or membranes of HEK293 cells transiently expressing hABHD6 or hABHD12

Radioenzymatic FAAH assay: [^{3}H]AEA → [^{3}H]EA + AA

Enzyme preparation	**[^{3}H]EA, % Total** Mean ± SD, n=3
Mock-HEK membrane	6.9 ± 0.5
hABHD6-HEK membrane	6.9 ± 0.7
hABHD12-HEK membrane	6.0 ± 0.6
Rat forebrain homogenate	13.5 ± 0.7

FAAH activity was determined using [^{3}H]AEA as the substrate as previously described (Saario et al., 2006). Briefly, HEK membranes (10 µg/tube) were incubated in 60 µl of 50 mM Tris-HCl buffer, pH 7.4 containing additionally 1 mM EDTA and 0.5 % (w/v) BSA at 37 °C for 10 min. After this, 40 µl of [^{3}H]AEA (2 µM final concentration) was added and the incubation continued for 10 min at 37 °C. The reaction was stopped by the addition of 400 µl ethyl acetate, followed by 100 µl of assay buffer. The samples were vortexed and liquid phases separated by centrifugation at 13000 rpm for 4 min. Radioactivity was determined from aliquots of water and organic phases. Data are expressed as the percentage of [^{3}H]AEA → [^{3}H]EA conversion from total radioactivity. Rat forebrain homogenate (18 µg/tube) served as a positive control. Data are mean ± SD from three independent experiments.

Fluorescent FAAH assay: DAMC → AMC + Decanoic acid

Enzyme preparation	**AMC Fluorescence** Mean ± SD, n=2
Mock-HEK lysate	113 ± 1
hABHD6-HEK lysate	110 ± 1
hABHD12-HEK lysate	113 ± 1
Mock-HEK membrane	115 ± 1
hABHD6-HEK membrane	116 ± 5
hABHD12-HEK membrane	114 ± 3
hFAAH-Cayman	927 ± 65
AMC-standard (0.625 µM)	1438 ± 66

FAAH activity was determined in 96-well-plate format using N-decanoyl 7-amino-4-methyl coumarin (DAMC, synthesized at the School of Pharmacy, University of Eastern Finland) as the substrate, following outlines of a previously published method (Kage et al., 2007). Briefly, the assay buffer was 50 mM Hepes-1 mM EDTA, pH 8.0. The wells contained 5 µl DMSO and 65 µl of the enzyme preparation (4.4 µg/well) diluted in assay buffer containing additionally 0.15 % (w/v) BSA. The reaction was started by adding DAMC (30 µl/well, 1 µM a final concentration, diluted in assay buffer containing additionally 7.1 % DMSO), the plate mixed and AMC fluorescence kinetically monitored for 90 min using a Tecan Infinite M200 plate reader with excitation and emission wavelengths of 360 and 430 nm, respectively. Data are expressed as raw fluorescence units, obtained with the gain setting 70 for this instrument. Human recombinant FAAH (hFAAH, Cayman Chemicals; Cat#10010183, 9.75 µg/well) served as a positive control. Relative fluorescence for the AMC standard is shown for comparative purposes. Data are mean ± SD from two independent experiments.

References:

Kage, K.L., Richardson, P.L., Traphagen, L., Severin, J., Pereda-Lopez, A., Lubben, T., Davis-Taber, R., Vos, M.H., Bartley, D., Walter, K., Harlan, J., Solomon, L., Warrior, U., Holzman, T.F., Faltynek, C., Surowy, C.S., and Scott, V.E. 2007 A high throughput fluorescent assay for measuring the activity of fatty acid amide hydrolase. *J. Neurosci. Methods* **161**: 47-54.

Saario, S.M., Poso, A., Juvonen, R.O., Järvinen, T., and Salo-Ahen, O.M. 2006 Fatty acid amide hydrolase inhibitors from virtual screening of the endocannabinoid system. *J. Med. Chem.* **49**: 4650-4656.