Methods

Materials. All chemicals used were obtained from Sigma-Aldrich. BODIPY FL casein and gelatin substrates were obtained from Invitrogen.

Animals and tissue samples. All experiments with animals were performed in accordance with protocols approved by the Bioethics Committee of the University of Patras in accordance with the European Legislation. The *Klk5*$^{-/-}$ animals were generated as described previously.[1] The *Spink5*$^{-/-}$ animals were kindly provided by Prof A.N. McKenzie (MRC Laboratory of Molecular Biology, Cambridge, UK). *Spink5*$^{-/-}$*Klk5*$^{-/-}$ animals were derived by two-stage crossing of *Spink5*$^{+/-}$ with *Klk5*$^{-/-}$. Skin specimens from 7 days old wt and *Spink5*$^{-/-}$ *Klk5*$^{-/-}$ were removed for preparation of biopsies and stored in O.C.T medium at -70^{o}C. 5 μm sections were cut with a cryotome (Leica).

Synthesis of the activity-based probe. 10 equiv of triethoxyphosphite (10.0 ml) were allowed to react with 1 equiv 11-bromoundecanoic acid (1,537 mg) for 24 hours at 170^{o}C in oil bath under reflux. The reaction mixture was partitioned between ethyl acetate and saturated $NaHCO_3$ in water. The aqueous layer was obtained and acidified with 1N HCl and extracted with ethyl acetate, washed with saturated NaCl and dried with Na_2SO_4. Solvent was evaporated and the product (11-diethoxyphosphoryl)undecanoic acid was recovered as a waxy white substance (yield 81%).

^{31}P-NMR ($CDCl_3$, 243 MHz) δ 36.10

^{1}H-NMR ($CDCl_3$, 600 MHz) δ 1.02-1.67 (m, 24H), 3.98 (m, 4H)

^{13}C-NMR δ 170.98, 61.37 (d, J=6.15 Hz), 34.09, 30.47, 29.19, 29.10, 29.08, 25.05, 20.88, 19.15, 18.20, 16.33 (d, J=6.3 Hz), 14.06

[ESI-MS: [M-H^{+}] m/z 321.39 calculated 321.18]

Then, 1.07 mmol (1 equiv) of (11-diethoxyphosphoryl)undecanoic acid in CH_2Cl_2 were treated with trimethysilyl bromide (0.17 ml, 1.2 equiv) at room temperature for 1 h and quenched with 5 ml 5% (w/v) $KHSO_4$. The mixture was partitioned between ethyl acetate and water. The organic layer was washed with saturated NaCl, dried with Na_2SO_4 and concentrated under reduced pressure to yield 11-(ethoxyhydroxyphosphoryl)undecanoic acid (yield 47%).

^{31}P-NMR (243 MHz, $CDCl_3$) δ 36.33

^{1}H-NMR (400 MHz, $CDCl_3$) δ 1.26-1.75 (m, 20H), 3.97 (m, 2H, CH_3CH_2OP) 2.22 (t, J=7.2 Hz, 2H, CH_2COOH)

^{13}C-NMR (151 MHz, $CDCl_3$) δ 173.32, 61.48 (d, J=6.60 Hz), 34.11, 30.50, 29.25, 29.17, 29.07, 25.10, 20.85, 19.22, 18.27, 16.41 (d, J=5.70 Hz), 13.99

The reaction was continued as described.[2] Briefly, a solution of 14 mg of 11-(ethoxyhydroxyphosphoryl)undecanoic acid in 0.8 ml CH_2Cl_2 at -78°C was treated dropwise with 0.042 ml (4 equiv) (diethylamino)sulfur trifluoride (DAST), brought to room temperature and stirred for 5 min. Then, one-half of the reaction volume of N-hydroxysuccinimide (10 equiv) dissolved in dimethyl formamide was added and the reaction was stirred for 10 min at room temperature. The reaction was partitioned between ethyl acetate and water and the organic layer was recovered, washed with saturate NaCl, dried with Na_2SO_4 and concentrated under reduced pressure to yield 11-(fluoroethoxyphosphoryl)-N-hydroxysuccinyl)undecamide (yield 74%).

^{31}P-NMR (243 MHz, $CDCl_3$) δ 36.33

^{1}H-NMR (600 MHz, $CDCl_3$) δ 1.09-1.80 (m, 23H), 2.33 (t, 2H, J=7.8 JHz), 2.81 (s, 4H succinimide), 4.08 (t, 2H, J=6.6 Hz)

^{13}C-NMR (151 MHz, $CDCl_3$) δ 169.55, 169.19, 169.02, 61.61 (d, J=6.30 Hz), 37.45, 31.92, 31.72, 31.62, 29.69, 29.65, 29.35, 28.74, 25.58, 25.46, 22.68, 16.44 (d, J=6.30 Hz), 14.10

2 mg of this compound prior to purification were treated with 1 equiv N-biotinyl-3,6,9-trioxaundecane-1,11-diamine in methanol for 10 min. The solvent was allowed to evaporate and the remaining residue was washed sequentially with hexane and diethylether. The final product (yield 51%) was dissolved in dimethyl sulfoxide [ESI-MS: $[M-H]^+$ m/z=695,42, calculated 696,37].

^{31}P-NMR (243 MHz, $CDCl_3$) δ 36.76

^{1}H-NMR (600 MHz, $CDCl_3$) δ 1.09-1.72 (m, 27H), 2.18-2.33 (m, 4H), 2.58 (d, 1H, J=8.4) 2.62 (s, 8H), 3.10-3.15 (m 8H), 3.97 (t, 2H, J=7.2 Hz), 4.06-4.11 (m, 2H), 5.02 (br s, 4 NH)

^{13}C-NMR (151 MHz, $CDCl_3$) δ 172.45, 169.19, 168.63, 65.98, 63.10, 63.07, 63.02, 61.74, 61.59, 61.51 (d, J=6.45 Hz), 60.48, 45.56, 41.17, 40.57, 34.32, 34.26, 34.19, 30.83, 29.56, 29.19, 29.10, 29.05, 28.66, 28.61, 28.55, 25.52, 25.37, 24.66, 24.49, 16.36 (d, J=5.4 Hz), 14.03

Activography. Skin cryosections (5 µm) mounted on glass slides were fixed in acetone for 10 min, air dried for 5 min and rehydrated in PBS for 5 min. The sections were incubated with PBS containing 3% H_2O_2 and subsequently with PBS containing 0.3% bovine serum albumin and 0.1% Triton X-100 to permeabilize the membranes. Sections were allowed to react with 10 µM ABP in PBS for 20 min at room temperature and then incubated with 1:500 dilution streptavidin-peroxidase polymer (S2438 Sigma) for 30 min at room temperature. After washing with PBS the reaction was visualized with the metal enhanced DAB substrate kit (Thermo Scientific). For pre-incubation experiments, the inhibitors were incubated prior to B24P at the indicated concentration for 2 h at room temperature.

***In situ* zymography.** Skin cryosections (5 µm) mounted on glass slides, were rinsed with 2% Tween-20 in PBS to permeabilize the membrane. Then, cryosections were incubated with 100 µg ml^{-1} BODIPY FL casein or BODIPY FL gelatin in 50 mM Tris-HCl, pH 8.0 for 3 and 1 h respectively at 37°C. Sections were rinsed with PBS, mounted with mowiol and visualized with a Leica confocal laser-scanning microscope (CLSM). Data were analyzed using ImageJ.

Detection of active KLK6 enzyme in cell supernatants. Conditioned media from SH-SY5Y cells transfected with the plasmid pcDNA3.1(+) driving the expression of active preproKLK6 R80Q cDNA or the inactive preproKLK6 S197A cDNA were concentrated with spin columns (Amicon). Concentrates were allowed to react with the ABP at 20 μM final concentration for 20 min at room temperature. Then, samples were resolved on SDS-PAGE under reducing conditions and transferred to PVDF membranes. The membranes were blocked with 5% non-fat dried milk in PBS for 30 min and incubated with 1:2,500 streptavidin-peroxidase polymer in PBS containing 0.05% Tween-20. Membranes were washed 4 times with PBS containing 0.05% Tween-20 for 5 min each and KLK6 enzyme-specific bands were visualized with Supersignal West Pico enhanced chemiluminescence kit (Thermo scientific).

Detection of recombinant KLK6. The indicated amounts of KLK6 were allowed to react for 20 min with the B24P in 20 μl final volume in PBS. The samples were processed as previously and detected with either the enhanced chemiluminescence kit (Thermo scientific) or the metal enhanced DAB substrate kit (Thermo Scientific).

Gelatin gel zymography. Conditioned media obtained as above were concentrated 50-fold and resolved on 12% SDS-PAGE containing 0.1% gelatin. Gels were washed twice with 50 mM Tris-HCl pH 7.5, 5 mM $CaCl_2$, 2.5% Triton X-100 for 15 min, 15 min with 50 mM Tris-HCl, pH 7.5, 5 mM $CaCl_2$, 0.1% Triton X-100, then, incubated in the latter buffer for 24 h and finally stained with Coomassie G-250.

References

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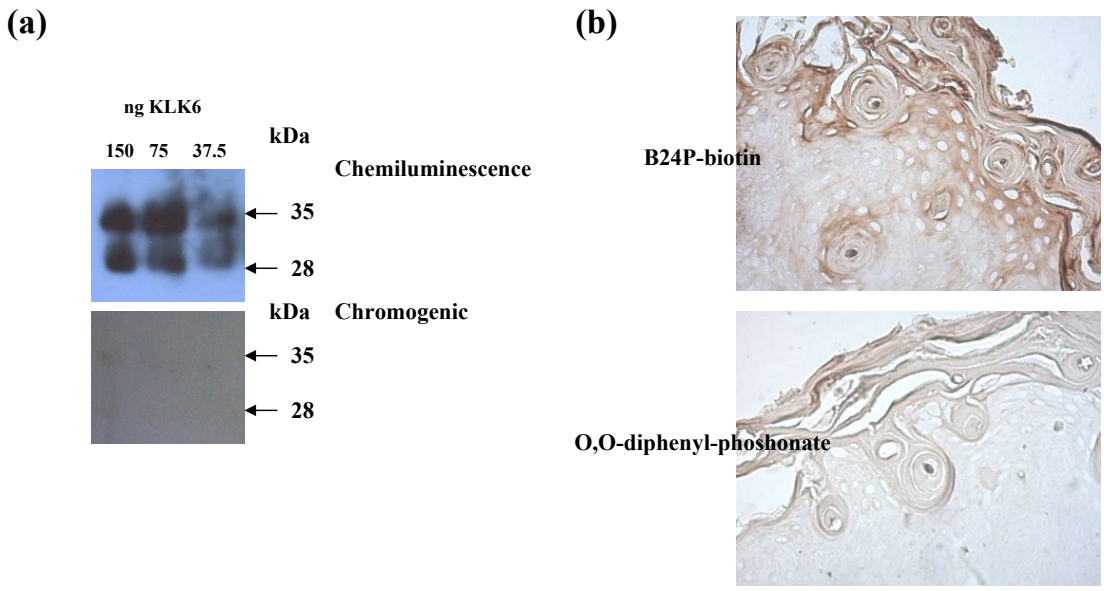
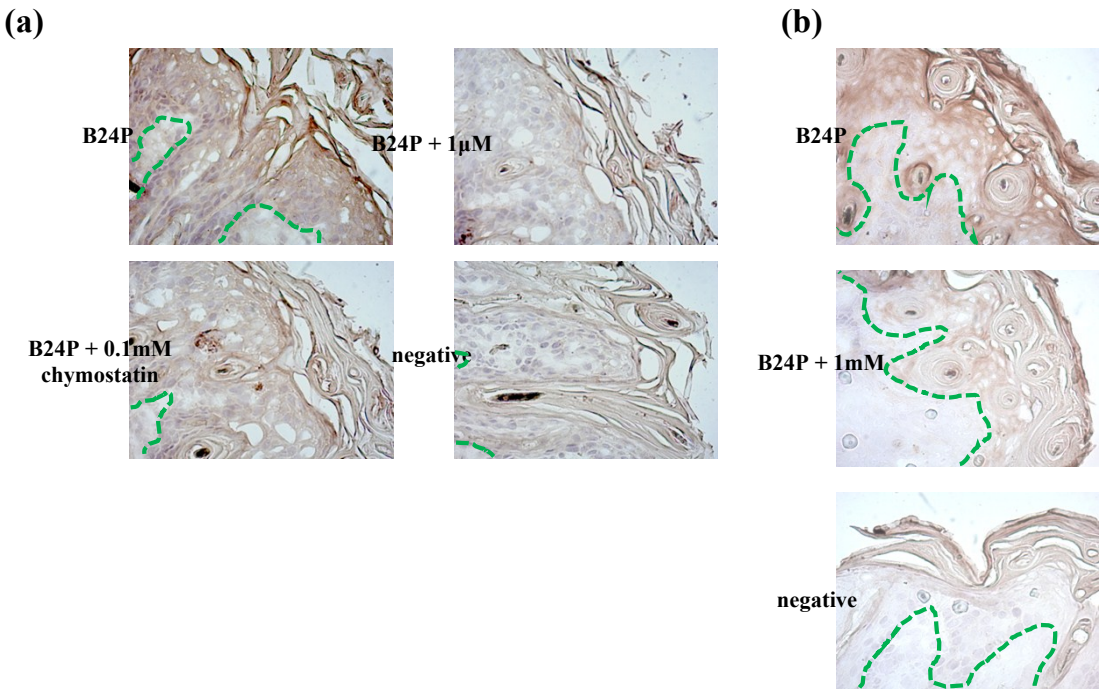
Y. Liu, M. P. Patricelli and B. F. Cravatt, Proc. Natl. Acad. Sci. U. S. A., 1999, **96**, 14694.



Supplementary Figure 1 (a) comparison of chemiluminescence (ECL) and chromogenic detection method for B24P. Known amounts of recombinant KLK6 were allowed to react with the probe, run on 12% SDS-PAGE, transferred on PVDF membranes, incubated with HRP-streptavidin and detected with ECL or chromogenic substrate. The chromogenic substrate only shows a very faint signal with 150 ng KLK6 while the signal with ECL is already saturated with 75 and 150 ng KLK6. (**b) comparison of B24P probe with the O,O-diphenyl organophosphate ester.** Within 20 min incubation of skin sections with the probes at room temperature only the B24P shows a signal. Sections from *Spink5*$^{-/-}$*Klk5*$^{-/-}$ mouse skin were used.



Supplementary Figure 2. (a) B24P does not react non-specifically with other protein present in skin tissue. Sections were pre-incubated with 1 μM aprotinin, a general serine protease inhibitor, that completely abolished the reaction with B24P. Pre-incubation with chymostatin significantly reduced the B24P labeling but did not completely inhibited, since chymostatin can only inhibit KLK7 chymotrypsin-like enzyme present in skin. However, the other KLKs that have trypsin-like activity remain active and free to bind B24P. **(b)** Comparison of the reaction rates between the B24P probe and the previously used O,O-diphenylphosphonate.[6] Within 20 min reaction at room temperature only B24P is able to give a positive signal, indicating a very rapid reaction. Both probes were used at 20 μM concentration on the slide. The skin sections were from *Spink5*$^{-/-}$*Klk5*$^{-/-}$ mouse. The green dashed line indicates the dermis-epidermis interface.