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Cortisol facilitates immune escape of human acute myeloid leukaemia cells by inducing latrophilin 1 expression

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23   Progression of acute myeloid leukaemia (AML) – the most severe blood/bone marrow cancer

24   – is determined by the ability of malignant cells to escape host immune surveillance.

25   However, the systemic regulation mechanisms underlying this phenomenon remain largely

26   unknown. In this study we have discovered a fundamental systemic biochemical strategy

27   which allows AML cells to employ physiological body systems to survive and escape

28   immune attack. We found that AML cells use a crucial human adrenal cortex hormone

29   (cortisol) to induce the expression of the neuronal receptor latrophilin 1 (LPHN1) which

30   facilitates exocytosis. This receptor interacts with the blood plasma protein FLRT3

31   (fibronectin leucine rich transmembrane protein 3) to cause secretion of the immune

32   suppressor galectin-9 which impairs the anti-cancer activities of cytotoxic lymphoid cells.

33   AML is a cancer of the blood and bone marrow which originates from self-renewing

34   malignant immature myeloid cells and rapidly becomes a systemic, and very often fatal,

35   malignancy[1]. AML cells employ body systems to produce factors required for their

36   proliferation/disease progression[2,3]. This includes employment of stem cell factor (SCF), a

37   major hematopoietic growth factor controlling AML progression and thus becoming highly

38   oncogenic[2,3]. Expression and release of SCF can be triggered by AML cells via cytokines

39   (e.g. interleukin-1β)[2]. Recent evidence clearly demonstrated that AML cells are also capable

40   of impairing the activities of cytotoxic lymphoid cells (e.g. natural killer (NK) cells and

41   cytotoxic T cells[4]). One of the biochemical mechanisms underlying this phenomenon lies in

42   the ability of AML cells to secrete the protein called galectin-9. This tandem type galectin

43   binds the immune receptor Tim-3 and induces a variety of intracellular and cell-to-cell

44   signalling events leading to the inactivation of NK cells as well as killing of cytotoxic T

45   cells[4,5]. We recently reported that the process of galectin-9 secretion in AML cells is

46   stimulated by the unique G protein-coupled receptor LPHN1, which normally functions in

47   neurons facilitating exocytosis[4,6]. LPHN1 is also found in haematopoietic stem cells (HSCs)

48   but its expression disappears at early stages of their maturation[4,7]. However, upon malignant

49   transformation, AML cells preserve their abilities to express LPHN1 and produce high levels

50   of galectin-9 and Tim-3, where the latter is involved in trafficking galectin-9 during the

51   secretion process (HSCs express neither galectin-9 nor Tim-3[4]).

52   It is currently unknown which molecular mechanisms trigger high levels of LPHN1

53   expression in primary human AML cells, and in general mechanisms of upregulation of

54   LPHN1 expression at the genomic level remain unclear. It is also unknown whether FLRT3,

55   a natural LPHN1 ligand[4,8], is present in human blood plasma and other tissues associated

56   with AML. Unravelling these mechanisms is crucial in order to understand the pathways that

57   control the ability of AML cells to protect themselves against cytotoxic lymphoid cells and

58   thus was the aim of the present study.

59   **Results and Discussion**

60   In order to investigate the effects of cortisol on LPHN1 transcription, we exposed primary

61   and THP-1 human AML cells, primary human HSCs and primary healthy human leukocytes

62   to 1 µM cortisol for 24 h followed by quantitative real-time PCR analysis of LPHN1 mRNA

63   levels. We found that all cell types, except primary healthy leukocytes, transcribed detectable

64   amounts of LPHN1 mRNA and in all these cases levels were significantly upregulated by

65   treatment with cortisol (Figure 1A). In both THP-1 and primary human AML cells LPHN1

66   protein levels were also clearly upregulated (Figure 1B and C). In contrast, primary human

67   healthy leukocytes did not express detectable amounts of LPHN1 protein and this was not

68   altered by the effects of cortisol (Figure 1D). Comparative analysis of LPHN1 protein

69   expression in primary human AML cells, THP-1 cells and primary human healthy leukocytes

70   is shown in Supplementary figure 1.

71 Cortisol treatments did not upregulate galectin-9 secretion in any of these cell types (Figure 1

72 B-D), suggesting that LPHN1 needs to be activated by a ligand to induce galectin-9 release.

73 Analysis of blood plasma levels of cortisol in AML patients vs healthy donors (samples were

74 collected at the same time of the day to avoid the influence of circadian dynamics)

75 demonstrated that its levels were significantly higher in the blood plasma of AML patients

76 compared to healthy donors (Figure 1E). Galectin-9 levels were also substantially higher in

77 AML patients (Figure 1F), which is in line with our previous observations[4]. Furthermore,

78 there was no correlation between cortisol and galectin-9 levels in the blood plasma of healthy

79 donors, while in AML patients there was a clear correlation (Figure 1G), suggesting that

80 galectin-9 secretion in this case might be linked to LPHN1 expression.

81 If LPHN1 is expressed on the surface of blood cells, it can also be shed by proteolysis and

82 therefore appear in the plasma. LPHN1 was immunoprecipitated, extracted and subjected to

83 Western blot analysis from blood plasma samples from AML patients using several LPHN1

84 antibodies. A clear fragment was detectable at around 67-68 kDa, smaller fragments were

85 detectable as well, but only in AML plasma, while in the blood plasma of healthy donors

86 there was no evidence of the presence of LPHN1 fragments (Figure 1H). These fragments

87 were also detectable by ELISA (Figure 1I, see Materials and Methods for description of the

88 ELISA format).

89 As reported before[4], we observed that exposure of THP-1 AML cells to 10 nM FLRT3 for 16

90 h resulted in a significant increase in galectin-9 secretion (Figure 2A). This effect was not

91 detectable in primary healthy human leukocytes (Figure 2A). Importantly, 1 h pre-exposure

92 of THP-1 cells to rabbit polyclonal antibody recognising LPHN1 (clone name RL1[9]) prior to

93 16 h treatment with 10 nM FLRT3 attenuated FLRT3-induced galectin-9 release, confirming

94 involvement of LPHN1 in this process (Figure 2A). The antibody employed specifically

95   recognised target molecules on the surface of THP-1 cells (Supplementary figure 2). We used

96   mouse neuroblastoma cells NB2A, which do not express LPHN1[10], as a negative control and

97   measured the interaction of the antibody with the cell surface using a Li-Cor on-cell assay as

98   described in the Materials and Methods (please see supplementary information). Exposure of

99   THP-1 cells to 1 µg/ml RL1 for 16 h did not affect galectin-9 secretion levels (data not

100   shown) suggesting that this antibody lacks a LPHN1 agonistic effect.

101   Interestingly, we found that blood plasma of both healthy donors and AML patients contains

102   approximately equal amounts of secreted FLRT3 (most likely by proteolytic shedding) with a

103   molecular weight of approximately 55 kDa (which corresponds to the molecular weight of

104   FLRT3 shed from the cell surface by proteinases[11]). Another specific band was seen at

105   around 27-28 kDa which most likely corresponds to a smaller cleavage fragment of the

106   FLRT3 extracellular domain (Figure 2B). The amounts of this smaller fragment were also

107   equal in blood plasma of healthy donors and AML patients (Figure 2B). To explore which

108   blood plasma-based ligands can induce galectin-9 secretion in AML cells we cultured THP-1

109   cells in RPMI-1640 medium containing antibiotics (as outlined in Materials and Methods –

110   see supplementary information) replacing 10 % foetal bovine serum (FBS) with blood plasma

111   from either healthy donors or AML patients. Cells were incubated for 16 h with or without 30

112   min pre-incubation with anti-FLRT3 antibody in order to neutralise FLRT3 activity.

113   Galectin-9 secretion levels were significantly higher in the presence of both sources of human

114   blood plasma compared to FBS (negative control). Anti-FLRT3 antibody attenuated galectin-

115   9 secretion (Figure 2C). Binding of LPHN1 and FLRT3 was further confirmed using SRCD

116   spectroscopy. We found that the two proteins interact with each other with high affinity,

117   inducing conformational change in both proteins, as seen from far UV synchrotron radiation

118   circular dichroism (SRCD) spectra (Figure 2D). This is further confirmation of the high

119 affinity interaction of LPHN1 and FLRT3 observed in previous studies[8] using different

120 techniques.

121 Taken together, our results demonstrate, for the first time, that cortisol upregulates LPHN1

122 expression at the transcriptional level, thus stimulating its translation in human AML cells.

123 AML leads to a decreased blood plasma glucose levels[5], which normally leads to

124 upregulation of secretion of corticotropin-releasing hormone (CTRH) by hypothalamus[12].

125 CTRH induces secretion of adrenocorticotropic hormone (ACTH) by pituitary gland[12].

126 ACTH upregulates cortisol production by the adrenal cortex[12]. Cortisol is then employed by

127 AML cells. In healthy human leukocytes cortisol is not capable of inducing LPHN1

128 transcription/translation, possibly because of gene repression. Interaction of AML cell-

129 derived LPHN1 with released FLRT3 available in blood plasma facilitates the secretion of

130 galectin-9. The latter protects AML cells against immune attack which could otherwise be

131 performed by NK cells as well as cytotoxic T cells (Supplementary figure 3). Importantly,

132 LPHN1 fragments are present in the blood plasma of AML patients but not in healthy donors.

133 These fragments were detectable by both Western blot analysis and ELISA, which indicates

134 the possibility of detection of these fragments for rapid AML diagnosis, although differential

135 verification tests have yet to be performed. Our results suggest a fundamentally novel

136 mechanism used by AML cells in order to progress the disease. They use a common

137 endogenous human hormone (cortisol) to induce LPHN1 expression by employing a widely

138 available ligand (FLRT3, which is always present in blood plasma) in order to escape host

139 immune surveillance. Thus, AML cells employ crucial functional systems of the human body

140 to support their survival and attenuate the anti-cancer activities of cytotoxic lymphoid cells.

141 Our work indicates that galectin-9 and secreted FLRT3 are the most promising targets for

142 anti-AML immune therapy.

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Materials and Methods are presented in Supplementary Information.

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Conflict of interest

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The Authors have no conflict of interest to declare

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Figure legends.

Figure 1. Cortisol induces LPHN1 expression in human AML cells and haematopoietic stem cells but not in primary healthy human leukocytes. Primary human AML, THP-1 and haematopoietic stem cells as well as primary healthy leukocytes were exposed to 1 µM cortisol for 24 h followed by analysis of LPHN1 gene transcription by quantitative real-time PCR (A) and Western blot analysis (B – primary AML cells, C – THP-1 cells and D – PHL). For PHL, lysates of LPHN1 overexpressing NB2A cells were used as a positive control. ELISA was used to measure secreted galectin-9 levels. Blood plasma of ten healthy donors and ten AML patients was collected at the same time of the day to ensure comparability of cortisol levels. Cortisol (E) and galectin-9 (F) levels were measured by ELISA and correlation between the levels of these two proteins was analysed (G). Soluble LPHN1 fragments were immunoprecipitated and detected by Western blot (H) and ELISA (I), as outlined in Materials and Methods. Images are from one experiment representative of four – six which gave similar results. Data represent mean values ± SEM of six – ten independent experiments. ; *p < 0.05; **p < 0.01; ***p < 0.01 vs. control.

Figure 2. FLRT3 induces galectin-9 secretion in AML cells in a LPHN1-dependent manner. THP-1 cells and PHL were exposed to 10 nM human recombinant FLRT3 for 16 h, followed by detection of secreted galectin-9 by ELISA. In THP-1 cells, the treatment was performed with or without 1 h pre-exposure to 1 µg/ml RL1 anti-LPHN1 polyclonal antibody

235   (A). The levels of released FLRT3 fragments were analysed in the blood plasma of healthy

236   donors and AML patients using Western blot (B). THP-1 cells were exposed for 16 h to 10%

237   blood plasma either from healthy donors or AML patients, with or without pre-treatment with

238   FLRT3 neutralising antibody. Levels of secreted galectin-9 were analysed using ELISA. (C).

239   Secondary structure and conformational changes of LPHN1, FLRT3 and the complex of the

240   two proteins were characterised using SRCD spectroscopy as outlined in Materials and

241   Methods (D). Images are shown from one representative experiment of four which gave

242   similar results. Data are shown as mean values ± SEM from four independent experiments;

243   *p < 0.05; **p < 0.01 vs. control.

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245   **Figure 1**
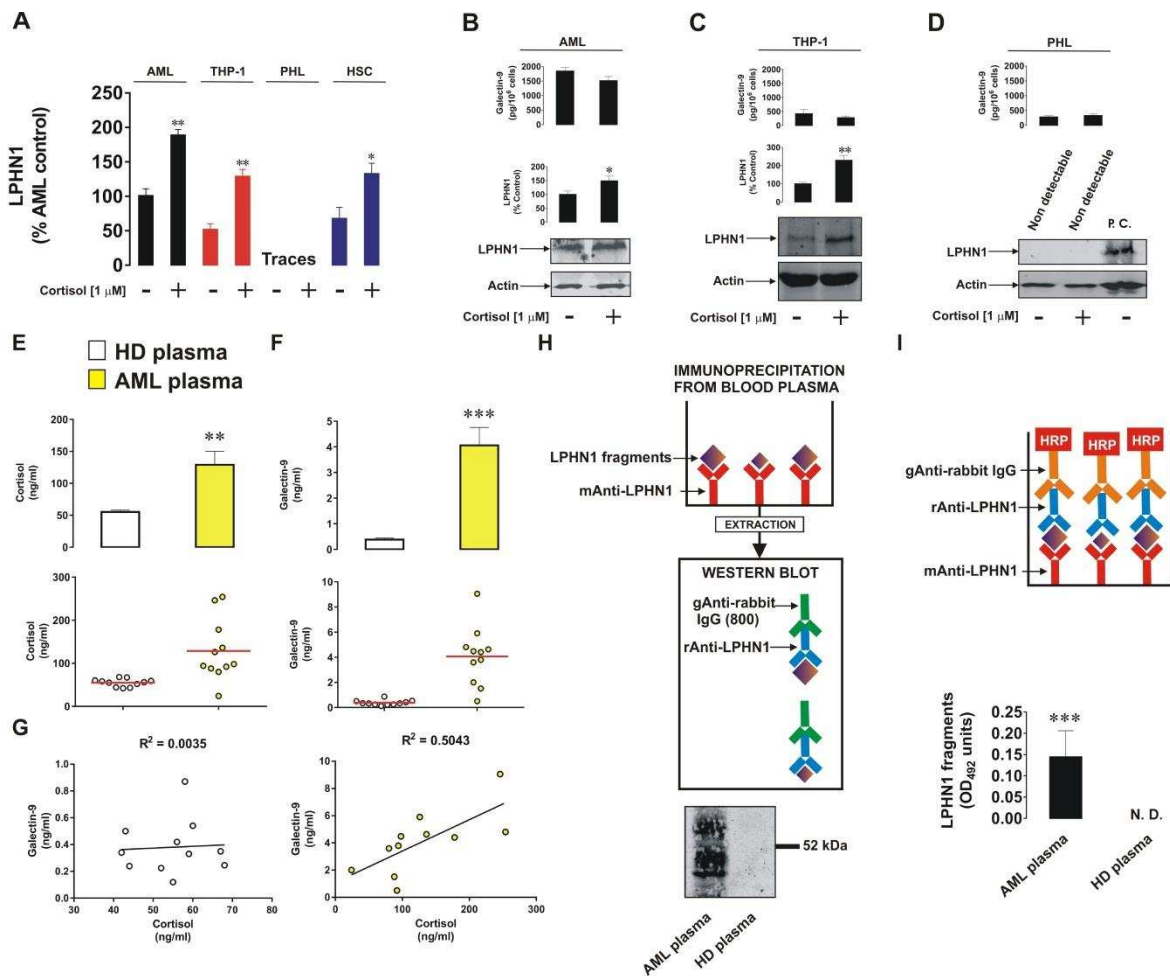
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247 **Figure 2**
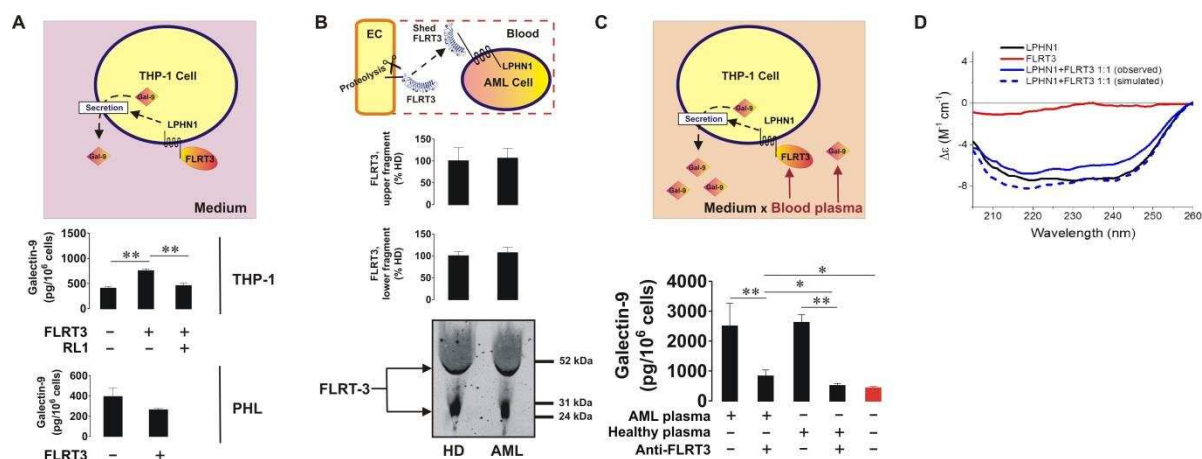
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251 **Supplementary Information**

252 **Cortisol facilitates immune escape of human acute myeloid leukaemia cells by inducing**

253 **latrophilin 1 expression**

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276 **Materials and Methods**

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278 **Materials**

279 RPMI-1640 medium, foetal bovine serum, supplements as well as basic laboratory chemicals

280 were purchased from Sigma-Aldrich (Suffolk, UK). Maxisorp™ microtitre plates were

281 obtained from Nunc (Roskilde, Denmark) and Oxley Hughes Ltd (London, UK). Human

282 recombinant FLRT3, mouse monoclonal antibody against LPHN1 and rabbit antibody against

283 FLRT3 were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). PAL1 and

284 RL1 rabbit polyclonal antibodies against LPHN1 were described previously[1,2]. Rabbit

285 antibody against native LPHN1 was obtained from Abcam (Cambridge, UK). Goat anti-

286 mouse and goat anti-rabbit fluorescence dye-labelled antibodies were obtained from LI-COR

287   (Lincoln, Nebraska USA). ELISA-based assay kits for the detection of galectin-9 were

288   purchased from Bio-Techne (R&D Systems, Abingdon, UK). A soluble extracellular

289   fragment of LPHN1, LPH-51, was produced and purified as described before[2]. Briefly, NB2a

290   cells stably expressing LPH-51 were cultured in serum-free medium for 48 h. 30 mL of the

291   medium was then incubated overnight at 4°C with 500 µL of anti-V5-antibody agarose

292   (Sigma-Aldrich). After incubation, the column was washed with 10 column volumes of PBS.

293   Bound protein was fractionally eluted with 5 volumes of elution buffer containing 50 mM

294   triethylamine and 150 mM NaCl (pH 12). The eluted protein was immediately neutralized

295   with 150 mM NaCl, 1 M HEPES (pH 7.2)[9]. The fractions were then analysed by Western

296   blotting and combined. The protein was concentrated using Amicon centrifugal ultrafiltration

297   units with a 30,000 molecular weight cut-off (Sigma-Aldrich). All other chemicals purchased

298   were of the highest grade of purity commercially available.

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301   **Cell lines and primary cells**

302   THP-1 human myeloid leukemia monocytes cells were obtained from the European

303   Collection of Cell Cultures (Salisbury, UK). Cells were cultured in RPMI 1640 medium

304   (R8758 – Sigma-Aldrich) with L-glutamine and sodium bicarbonate, liquid, sterile-filtered,

305   suitable for cell culture) supplemented with 10% foetal bovine serum, penicillin (50 IU/ml)

306   and streptomycin sulphate (50 µg/ml). Untransfected mouse neuroblastoma cells (NB2A) and

307   those overexpressing the full-size LPHN1 (LPH-42) were handled as described earlier[9].

308   Primary human AML cells were obtained from the sample bank of the University Medical

309   Centre Hamburg-Eppendorf (Ethik-Kommission der Ärztekammer Hamburg, reference:

310 PV3469). Cells were incubated in IMDM medium containing 15% BIT 9500 serum

311 substitute, 100 µM mercaptoethanol, 100 ng/ml stem cell factor (SCF), 50 ng/ml FLT3, 20

312 ng/ml G-CSF, 20 ng/ml IL-3, 1 µM UM729 and 500 nM stemregenin 1 (SR1) as described

313 before[3].

314 **Primary human blood plasma samples**

315 Blood plasma from healthy donors was generated by centrifugation of peripheral blood

316 provided by the National Health Blood and Transfusion Service (NHSBT, UK) following

317 ethical approval (REC reference: 16-SS-033). Primary human AML plasma samples were

318 obtained from the sample bank of University Medical Centre Hamburg-Eppendorf (Ethik-

319 Kommission der Ärztekammer Hamburg, reference: PV3469)[4].

320 **Western blot analysis**

321 LPHN1 protein levels were analysed using Western blotting. β-actin staining was used to

322 confirm equal protein loading as described previously (4, 6, 14). LI-COR goat secondary

323 antibodies (dilution 1:2000), conjugated with fluorescent dyes, were used in accordance with

324 manufacturer's protocol to visualise target proteins (using a LI-COR Odyssey imaging

325 system). Western blot data were quantitatively analysed using Odyssey software and values

326 were subsequently normalised against those of β-actin[1,4,5].

327 **Enzyme-linked immunosorbent assays (ELISAs) and immunoprecipitation**

328 Secreted galectin-9 was measured by ELISA using R&D Systems kits according to

329 manufacturer's protocol. Plasma cortisol was measured by ELISA using the Salimetrics assay

330 kit according to the manufacturer's protocols (Salimetrics, Suffolk, UK.)

331 For immunoprecipitation of LPHN1 fragments from human blood plasma, plates were coated

332 with mouse monoclonal LPHN1 antibody following the principle and protocol described

333   previously[5]. This was followed by blocking with 2% BSA. Samples were then applied and

334   incubated for at least 4 h at room temperature after which plates were extensively washed

335   with Tris buffered saline with Tween-20 (TBST). Glycine-HCl pH lowering buffer (pH 2.0)

336   was then applied to extract the bound proteins. Extracts were mixed with equal volumes of

337   lysis buffer (pH 7.5) and with 4× sample buffer for SDS-PAGE at a ratio of 1:3. Samples

338   were then subjected to Western blot analysis (5), using rabbit PAL1 anti-LPHN1 antibody.

339   Alternatively, PAL1 antibody was applied as detection antibody, followed by visualisation

340   using HRP-labelled anti-rabbit secondary antibody (Abcam, Cambridge, UK).

341   **On cell assays**

342   We employed a standard LI-COR on cell assay to characterise interaction of RL-1 antibody

343   with the surface of THP-1 cells[4,5].

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346   **Synchrotron radiation circular dichroism (SRCD) spectroscopy**

347   Human recombinant LPHN1 and FLRT3 were analysed, either alone or in combination with

348   each other, using SRCD spectroscopy at beamline B23, Diamond Light Source (Didcot, UK).

349   (The B23 is equipped with a highly collimated microbeam allowing the use of small aperture

350   long path length microcuvettes[6-9]). SRCD measurements were carried out using 0.01 µM

351   sample of soluble LPH-51 in a 1 cm path length cell of 3 mm aperture diameter using a

352   Module B instrument with 1 nm increment, 1 s integration time and 1.2 nm bandwidth at 23

353   °C. The cuvette capacity was 60 µl. Titration experiments were conducted as described

354   previously[3] using standard far-UV measurements.

355   **Statistical analysis**

356 Each experiment was performed at least three times and statistical analysis was conducted

357 using a two-tailed Student's t-test, where appropriate. Multiple comparisons were performed

358 using an ANOVA test. Post-hoc Bonferroni correction was applied. Statistical probabilities

359 (p) were expressed as * where $p<0.05$; **, $p<0.01$ and *** when $p<0.001$. Coefficient of

360 determination (R^2) was calculated using GraphPad Prism software in order to assess

361 correlation.

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397 **Supplementary Figures**

398

399 **Cortisol facilitates immune escape of human acute myeloid leukaemia cells by inducing**

400 **latrophilin 1 expression**

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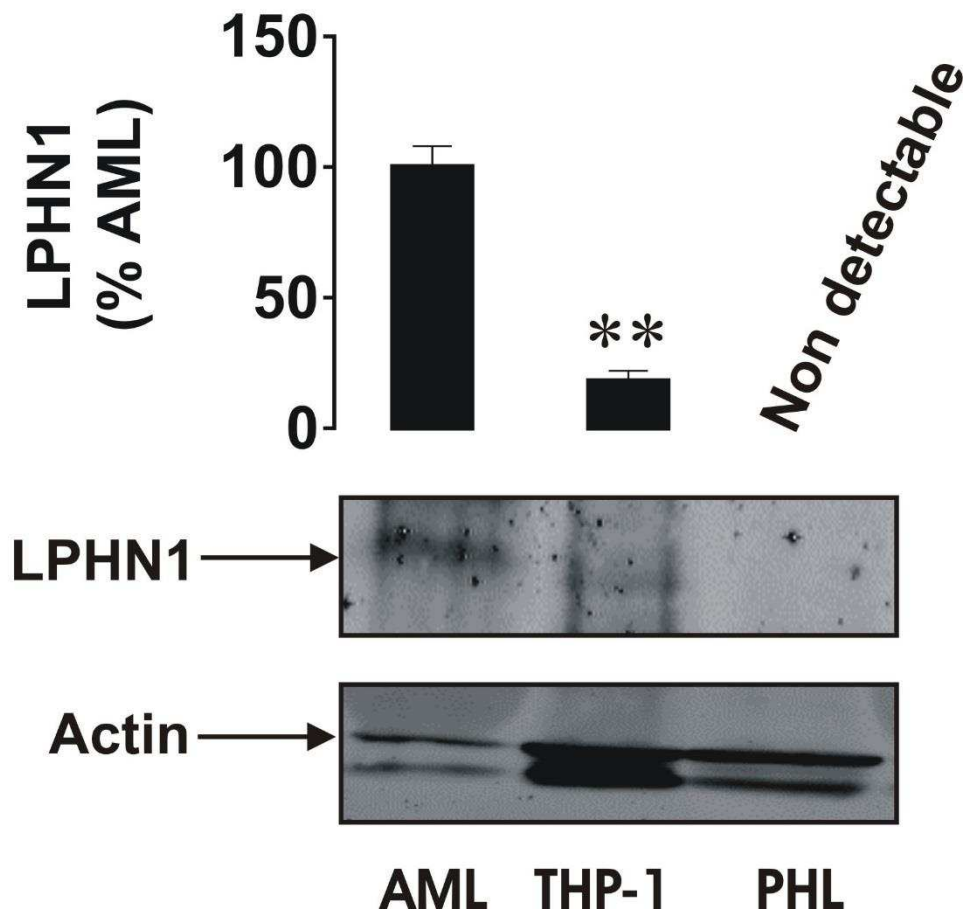
Supplementary figure 1. Comparative analysis of LPHN1 protein expression in primary human AML, THP-1 cells and PHL. Lysates of each cell type were subjected to Western blot analysis as outlined in the Materials and Methods. Images are from one experiment representative of three which gave similar results. Data represent mean values ± SEM of three independent experiments; **p < 0.01 vs. AML cells.
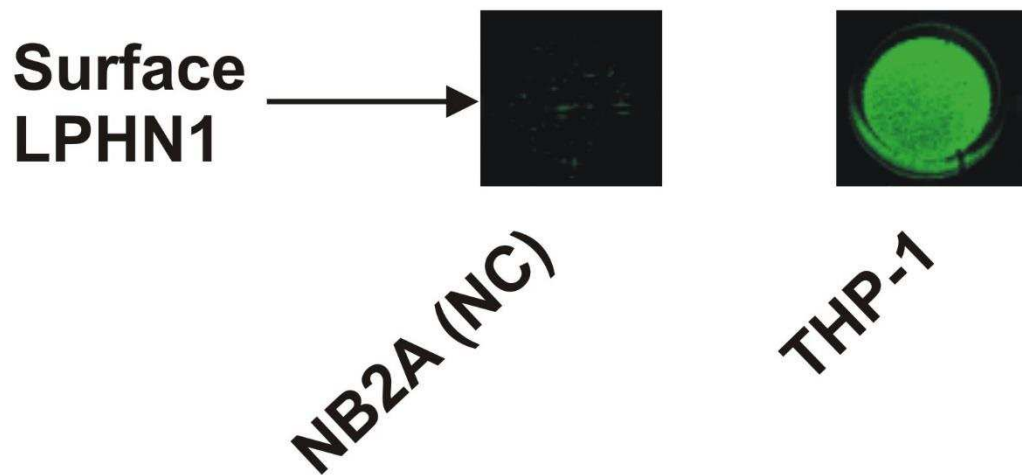
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430 **Supplementary figure 2. Cell surface detection of LPHN1.** Wild type NB2A cells
431 (negative control) and THP-1 cells were subjected to an on-cell assay using anti-rabbit Li-Cor
432 secondary antibody. Images are from one experiment representative of three which gave
433 similar results.

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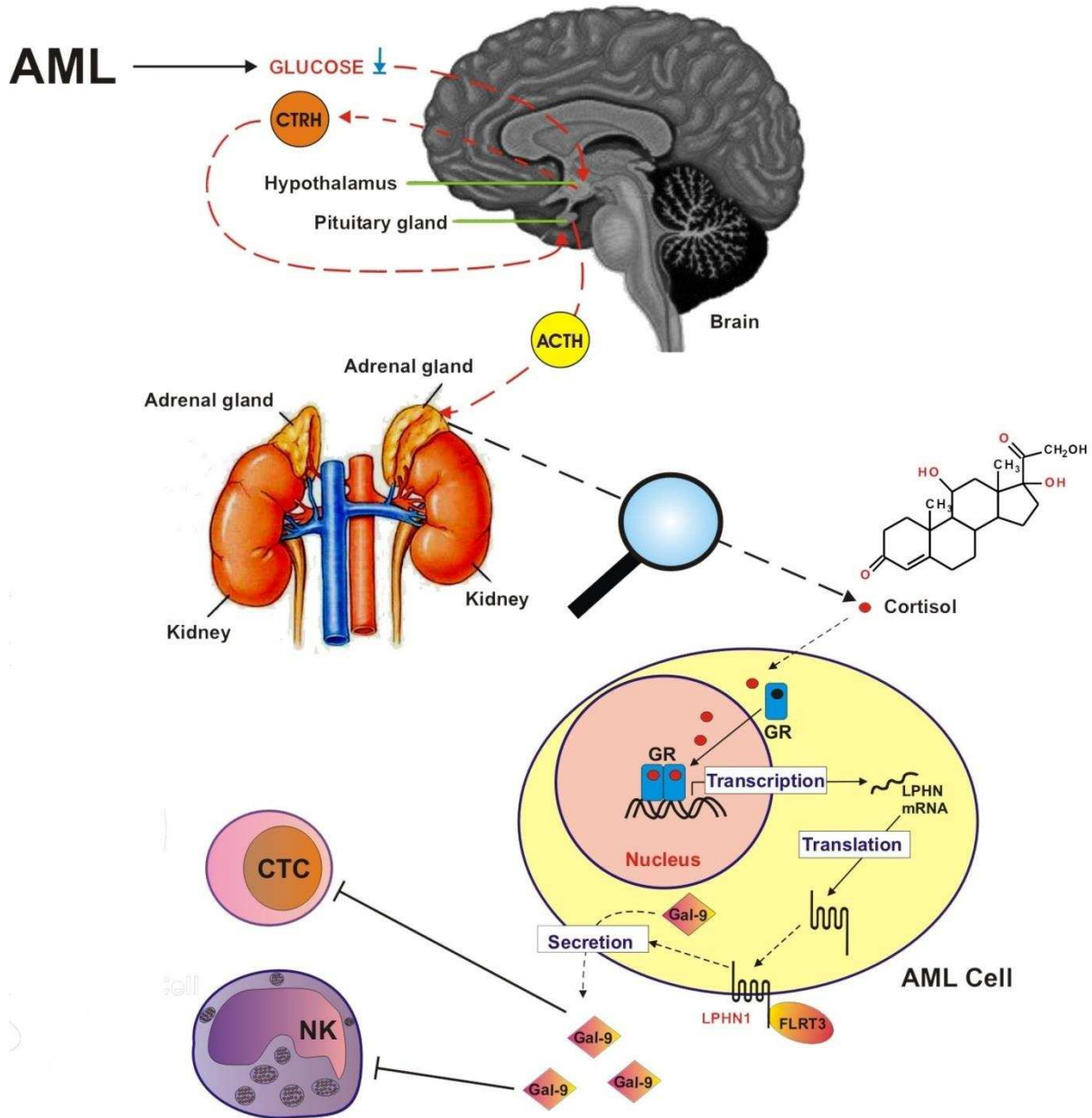
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Supplementary figure 3. Physiological cross-links leading to cortisol-induced upregulation of LPHN1 expression in AML cells followed by facilitation of galectin-9 secretion in a FLRT3-dependent manner. AML is associated with a decreased blood plasma glucose levels, which normally leads to upregulation of secretion of corticotropin-releasing hormone (CTRH) by hypothalamus. CTRH induces secretion of adrenocorticotropic hormone (ACTH) by pituitary gland. Secreted ACTH upregulates cortisol production by the adrenal cortex, thus leading to cortisol-induced upregulation of LPHN1 levels in AML cells.

461   Galectin-9, secreted in FLRT3-LPHN1-dependent manner attenuates anti-cancer activity of
462   cytotoxic T cells (CTC) and NK cells.

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