

## EXTRACELLULAR ATP ACTIVATES HYALURONAN SYNTHASE 2 (*HAS2*) IN EPIDERMAL KERATINOCYTES VIA P2Y<sub>2</sub>, CA<sup>2+</sup>-SIGNALING, AND MAPK PATHWAYS<sup>1,2</sup>

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<sup>2</sup>Abbreviations used:

CaMKII = Ca<sup>2+</sup>/calmodulin-dependent protein kinase II

CREB = calcium response element binding protein

HA-ELSA = hyaluronan enzyme-linked sorbent assay

HAS = hyaluronan synthase

MAPK = mitogen-activated protein kinase

P1, P2X, P2Y = purinergic (nucleotide and nucleoside) receptors

PKC = protein kinase C

STAT = signal transducer and activator of transcription

UDP-GlcNAc = uridine diphosphate N-acetyl glucosamine

UVB = ultraviolet B radiation

**ABSTRACT**

Extracellular nucleotides are used as signaling molecules by several cell types. In epidermis, their release is triggered by insults such as ultraviolet radiation, barrier disruption and wounding and by specific nerve terminals firing. Increased synthesis of hyaluronan, a ubiquitous extracellular matrix glycosaminoglycan, also occurs in response to stress, leading to the attractive hypothesis that nucleotide signaling and hyaluronan synthesis could also be linked.

In HaCaT keratinocytes ATP caused a rapid and strong but transient activation of hyaluronan synthase 2 (*HAS2*) expression via PKC-, CaMKII-, MAPK- and CREB-dependent pathways by activating the purinergic P2Y<sub>2</sub> receptor. Smaller but more persistent upregulation of *HAS3* and *CD44*, and delayed upregulation of *HAS1* were also observed. Accumulation of peri- and extracellular hyaluronan followed 4-6 h after stimulation, an effect further enhanced by the hyaluronan precursor glucosamine. AMP and adenosine, the degradation products of ATP, markedly inhibited *HAS2* expression and, despite concomitant upregulation of *HAS1* and *HAS3*, inhibited hyaluronan synthesis. Functionally, ATP moderately increased cell migration, whereas AMP and adenosine had no effect.

Our data highlight the strong influence of adenosinergic signaling on hyaluronan metabolism in human keratinocytes. Epidermal insults are associated with extracellular ATP release, as well as rapid upregulation of *HAS2/3*, *CD44* and hyaluronan synthesis, and we show here that the two phenomena are linked. Furthermore, as ATP is rapidly degraded, the opposite effects of its less phosphorylated derivatives facilitate a rapid shut-off of the hyaluronan response, providing a feedback mechanism to prevent excessive reactions when more persistent signals are absent.

## INTRODUCTION

ATP is the classical energy currency of live cells. In addition, it serves as an important signal transmitter in various tissues, including the epidermis [1,2], and between tissues, particularly the nervous system and target organs [3]. It is released in response to mechanical stimulation [4], temperature stress [2], and chemical receptor stimulation [5] by direct cell lysis, exocytosis, or secretion via e.g. connexin hemichannels and pannexin channels [6]. ATP is also released after insults such as ultraviolet (UV) radiation [7,8] and tissue wounding or skin barrier disruption [9,10]. Additionally, extracellular ATP mediates inflammatory signals [11,12] and pain sensation (reviewed in [13]).

ATP is released into the extracellular space at relatively high concentrations and degraded rapidly to ADP, AMP, and further to adenosine [14], which, except for AMP, are known to activate specific receptors even in a self-sustaining manner [15]. ATP signals through both the ionotropic P2X (cation channels), and the metabotropic P2Y receptors, which activate intracellular second messengers via specific G protein activation. The initial discovery of the role of purinergic signaling in rapid responses regulating neurotransmission and secretion was later followed by observations that extracellular nucleotides also modulate long-term or trophic effects including cell proliferation, differentiation and migration (reviewed in [16]). There are seven functional P2X receptors (P2X<sub>1-7</sub>), and eight P2Y receptors (P2Y<sub>1-2</sub>, P2Y<sub>4</sub>, P2Y<sub>6</sub> and P2Y<sub>11-14</sub>; [3]). In addition, another family of metabotropic P1 receptors with four distinct members (A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub>) relays the effects of extracellular adenosine [3]. Besides ATP, the versatile P2Y family also utilizes other nucleotides and their derivatives: the main ligands also include UTP (P2Y<sub>2</sub>, P2Y<sub>4</sub>), UDP (P2Y<sub>6</sub>) and UDP-glucose (P2Y<sub>14</sub>; see [3,17] for more complete listings of the known agonists).

ADP and adenosine utilize the metabotropic P2Y and P1 receptors, respectively. The ADP receptor P2Y<sub>1</sub> and the ATP receptors P2Y<sub>2</sub> and P2Y<sub>11</sub> are coupled to G<sub>q/11</sub> proteins. They activate PLC, resulting in the formation of diacylglycerol and IP<sub>3</sub> leading directly and indirectly, via Ca<sup>2+</sup> release from the endoplasmic reticulum through IP<sub>3</sub> receptor activation, to activation of PKC. The

ADP receptors P2Y<sub>12</sub> and P2Y<sub>13</sub> couple with G<sub>i/o</sub> to inhibit adenylyl cyclase and cAMP production [18,19]. Keratinocytes express several purinergic receptors from the P2Y and P2X families [7,11,20]. Of the P1-receptors, A<sub>2B</sub> is the main subtype [21,22].

Interestingly, it appears that the expression of P2 receptors may depend on the differentiation status of the keratinocytes, as P2Y<sub>1</sub> and P2Y<sub>2</sub> are found mainly in the basal cell compartment, while P2X<sub>5</sub> is located in the basal and spinous layers, and P2X<sub>7</sub> is found in the stratum corneum [23]. Functionally, P2Y<sub>2</sub> is then associated with proliferation, P2X<sub>5</sub> with differentiation and P2X<sub>7</sub> with apoptosis.

The potency of ATP on the different purinergic receptors varies with measured EC<sub>50</sub> values ranging from 0.1 μM for P2X<sub>1</sub> to as high as 2-4 mM for P2X<sub>7</sub> [24]. For the P2Y family, EC<sub>50</sub> values are in the micromolar range for ATP, whereas nanomolar ADP may be effective, depending on the receptor [17]. However, determining concentration dependency is difficult due to the quick metabolism of the agonists as well as the differential expression levels of the receptors, which may also change rapidly in response to physiological stimuli. The potency data also depends on the endpoint measured: e.g. it has been noted, that adenosine is least effective in modulating cAMP levels through the P1 receptor A<sub>2B</sub>, whereas all the family members produce similar responses with respect to MAP kinase activation (reviewed in [25]).

Hyaluronan is composed of repeating disaccharides of D-glucuronic acid and N-acetyl-D-glucosamine. This linear polysaccharide is synthesized at the inner leaflet of the plasma membrane and translocated into the extracellular matrix by the synthesizing enzymes [26]. The three highly homologous mammalian hyaluronan synthases (HAS1-3; [27,28]) differ in tissue distribution, expressional regulation and synthetic activity [29,30]. Hyaluronan can remain associated pericellularly via the synthases or specific receptors such as CD44, or be released to associate with other matrix molecules [31].

Hyaluronan is abundant in the dermal connective tissues and the epidermis [32]. Epidermal hyaluronan synthesis is rapidly upregulated after tissue wounding [33], disruption of the



permeability barrier [34] and UVB exposure [35]. As extracellular nucleotides are similarly released after tissue injury, it is relevant to ask, whether these processes could be linked. Previous data on the effects of nucleosides and nucleotides on hyaluronan metabolism are scarce, but adenosine is known to upregulate *HAS1* expression and hyaluronan secretion in gingival fibroblasts [36] and smooth muscle cells [37], while UDP-Glc, UTP and UDP enhance *HAS2* expression and hyaluronan synthesis in keratinocytes [38,39].

In this work we wanted to explore the role of ATP and its metabolites in hyaluronan metabolism and signaling in human keratinocytes. Our data indicate that extracellular ATP and its degradation products AMP and adenosine are potent regulators of *HAS2*, enabling a rapid launch and shut-off of hyaluronan synthesis, depending on nucleotide release and degradation. Furthermore, the upregulation of *HAS2* seemed to depend heavily on the activation of the purinergic P2Y<sub>2</sub> receptor and downstream signaling cascades involving the calcium-activated PKC, CaMKII and CREB as well as the MAP kinases p38 and pERK. Overall, our data shed light on the complex regulation of HA synthesis in keratinocytes under simulated stress conditions.

## MATERIALS AND METHODS

**Cell culture** - HaCaT, a spontaneously immortalized human epidermal keratinocyte cell line developed by Fusenig and colleagues [40], were cultured in DMEM (Sigma-Aldrich, Inc., St. Louis, MO) containing 10% FBS (GE Healthcare Life Sciences/HyClone, Logan, UT), 2 mM L-glutamine (EuroClone, Milan, Italy), 50 units/ml penicillin and 50 µg/ml streptomycin (EuroClone). The same base medium was used when treating the cells with the different nucleotides, siRNAs and chemical inhibitors, unless otherwise specified.

**Nucleotide treatments** - ATP, ATP $\gamma$ S,  $\beta$  $\gamma$ -methylene ATP, ADP, AMP and adenosine (Sigma-Aldrich) were dissolved in H<sub>2</sub>O and stored as stock solutions at -20°C. The nucleotides were applied at 100 µM unless otherwise specified for 15 min-24 hours.

**Signaling modulators** - A two-hour pretreatment was used with the CaMKII inhibitor KN93 (25 µM; Calbiochem/Merck Millipore, Darmstadt, Germany), the JAK2/EGFR inhibitor AG490 (30 µM; Sigma-Aldrich) and the STAT3 inhibitor IX/Cpd188 (50 µM; Calbiochem/Merck Millipore). A 30-min pretreatment before adding ATP was applied with the MEK inhibitor PD98059 (0.5 µM; Calbiochem/Merck Millipore), the PKC inhibitor bisindolylmaleimide I (BIM, 10 µM; Calbiochem/Merck Millipore), the CREB inhibitors KG501 (naphthol AS-E phosphate, 25 µM; Sigma-Aldrich) and naphthol AS-BI phosphate (25 µM; Santa Cruz Biotechnology, Inc., Dallas, TX), the P2Y<sub>1</sub> inhibitor MRS2179 (5-30 µM; Tocris Bioscience, Bristol, UK), the P2Y<sub>11</sub> inhibitor NF340 (10 µM; Tocris Bioscience), and the p38 inhibitor BIRB796 (2 µM; Axon Medchem BV, Groningen, The Netherlands). With pertussis toxin (PTX, 100 ng/ml; Sigma-Aldrich) a 17-hour preincubation was used. BIM, naphthol AS-BI-phosphate, NF340, MRS2179, KN93 and PTX were dissolved in water, and the STAT3 inhibitor IX, AG490, PD98059, KG501 and BIRB796 in DMSO. Equal amounts of these solvents were added to the control cultures, where necessary.

**siRNA-treatments** - P2Y<sub>2</sub>-targeted siRNAs were obtained from Thermo Fisher Scientific (Invitrogen/Thermo Fisher Scientific, Waltham, MA) and the scrambled control siRNA from

Eurogentec (Eurogentec/Kaneka, Osaka, Japan). Subconfluent cultures were transfected with 40 nM siRNA (a mix of two specific sequences targeting *P2Y2*) using Lipofectamine RNAiMAX (Invitrogen/Thermo Fisher Scientific) according to the manufacturer's instructions. The transfection medium was removed after 4 h and replaced with regular culture medium. Total RNA was collected 2 days after the transfection and a 2-h incubation with ATP. Efficacy of the knockdown was confirmed by qRT-PCR.

**RNA extraction and qRT-PCR** - Total RNA was extracted with Eurozol (EuroClone). cDNA synthesis with 1 µg of total RNA as a template was performed using a Verso™ cDNA kit (Invitrogen/Thermo Fisher Scientific). Quantitative RT-PCR was run on a Stratagene Mx3000P thermal cycler (Agilent, La Jolla, CA), using the FastStart Universal SYBR Green Master with ROX (Roche, Basel, Switzerland). The cycling conditions were as follows: preincubation for 10 min at 95°C followed by 40 cycles of 15 s denaturation at 95°C, 60 s annealing at a primer specific temperature and 60 s elongation at 72°C. Gene-specific amplification was confirmed by a melt curve analysis. The primers for the transcripts analyzed and the respective annealing temperatures are presented in Supplementary Table S1. Fold inductions were calculated using the formula  $2^{(-\Delta\Delta C_t)}$ , where  $\Delta\Delta C_t$  is  $\Delta C_{t(\text{sample replicate})} - \Delta C_{t(\text{non-treated replicate})}$ ,  $\Delta C_t$  is  $C_{t(\text{gene of interest})} - C_{t(ARPO)}$  and  $C_t$  is the cycle at which the detection threshold is crossed.

**Western blotting** - Proteins were extracted by incubating the cultures on ice for 30 min with RIPA lysis buffer (150 mM NaCl, 50 mM Tris, 1% Nonidet P-40, 0.5% sodium deoxycholate and 0.1% SDS) containing 10 mM NaF, 1% phosphatase inhibitor cocktail 2, and 0.5% protease inhibitor cocktail (Sigma-Aldrich). Protein content was measured using the Bradford assay [41]. Samples containing 6.5-7.5 µg total protein were resolved by 10% SDS-PAGE followed by transfer onto nitrocellulose (Amersham™ Protran™ 0.45 µm NC; GE Healthcare Life Sciences, Little Chalfont, UK) by a 2-3 mA/cm<sup>2</sup> constant current with a Fastblot B43 semidry blotter (Biometra GmbH, Göttingen, Germany). The membrane was rinsed briefly with Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, pH 7.4), blocked with 1-5% BSA-TBS for 30 min, and incubated

overnight at 4°C on a rolling mixer with the following primary antibodies: phospho-STAT3 (Tyr<sup>705</sup>) or phospho-STAT3 (Ser<sup>727</sup>) diluted 1:1000 in 1% BSA-TBS, phospho-CREB (Ser<sup>133</sup>) diluted 1:1000 in 5% BSA-TBS and phospho-p38 diluted 1:1000 in 5% BSA-TBS (all from Cell Signaling, Danvers, MA), phospho-ERK1/2 diluted 1:200 in 2% BSA-TBS (Santa Cruz Biotechnology, Inc.) and either mouse or rabbit  $\beta$ -actin diluted 1:4000 in 2% BSA-TBS (Sigma-Aldrich). After washes with 0.1% Tween 20-TBS, the membranes were incubated with the secondary antibodies DyLight anti-rabbit 680 or 800 diluted 1:3000 in 2% nonfat milk-TBS (for pSTAT3) or 1:4000 in 2% BSA-TBS (for pCREB, phospho-p38 and rabbit  $\beta$ -actin) and anti-mouse 680 or 800 diluted 1:2000 in 2% nonfat milk-TBS (for pERK) or 1:4000-1:5000 in 2% BSA-TBS (for mouse  $\beta$ -actin; all secondaries from Pierce, Rockford, IL).  $\beta$ -actin was probed separately after a 10-min stripping with 0.2 M NaOH. Protein bands were visualized and quantified with an Odyssey<sup>®</sup> infrared imaging system (LI-COR Biosciences, Lincoln, NE), normalizing all protein intensities to  $\beta$ -actin.

**HA-ELSA** - For hyaluronan assays, HaCaT cells were cultured on 12- or 24-well plates and treated with the respective nucleotides (100  $\mu$ M). Here, culture medium supplemented with 1% FBS was used for the entire duration of the nucleotide treatments (4-24 h). Additionally, some wells were supplemented with 1 mM glucosamine to provide an excess of the hyaluronan precursor UDP-GlcNAc. The media and trypsinates (representing extra- and pericellular hyaluronan, respectively) were collected for hyaluronan quantification after 4, 6 or 24 h. Cells were released with trypsin and counted for data normalization. A sandwich-type enzyme-linked sorbent assay for hyaluronan measurement (HA-ELSA) was performed as described previously [42].

**Quantification of hyaluronan nucleotide sugar precursors by HPLC** - HaCaT cells on 10-cm dishes were treated with 100  $\mu$ M ATP for 1.5, 3, or 4.5 hours. Cells were extracted and nucleotide sugars measured as described previously [43]. Briefly: The cells were washed twice with and scraped into ice-cold PBS, sonicated and centrifuged. The samples were purified with Superclean Envi-Carb SPE-tubes (Sigma-Aldrich) followed by vacuum centrifugation. The dried samples were dissolved in water and analyzed by anion exchange HPLC (CarpoPac<sup>™</sup> PA1 column;

Dionex/Thermo Fisher Scientific) as described previously [43]. Total protein was measured with a Pierce BCA kit (Thermo Fisher Scientific) for normalization of the data.

**Hyaluronan staining** - For visualization of cell-associated hyaluronan, the cells were fixed for 20 min in 2% paraformaldehyde, permeabilized and blocked for 10 min using 0.1% Triton X-100 in 0.1 M phosphate buffer pH 7.0 (PB), containing 1% BSA. The cultures were incubated overnight at 4°C with a biotinylated hyaluronan binding complex (bHABC, 3 µg/ml; prepared in-house as described previously [44]) and subsequently for 1 h at room temperature with the ABC reagent (1:200, VECTASTAIN Elite ABC Kit; Vector Laboratories, Burlingame, CA). For visualization of the bound probe, the cultures were incubated for 5 min in 0.05% diaminobenzidine (DAB; Sigma-Aldrich) and 0.03% hydrogen peroxide in PB. The nuclei were counterstained with Mayer's hematoxylin. The stained cultures were viewed and imaged with a Zeiss Axio Imager M2 light microscope (Carl Zeiss Microimaging GmbH, Jena, Germany).

For dual stainings and confocal imaging, the anti-CD44 antibody Hermes 3 (a kind gift from professor Sirpa Jalkanen, University of Turku) was added to the bHABC solution at a 1:100-dilution. FITC-labeled anti-mouse IgG (1:200) and TR-streptavidin (1:1000; both from Vector Laboratories) were the secondary reagents, respectively. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The stainings were photographed using a 40x NA 1.3 oil objective on a Zeiss Axio Observer inverted microscope equipped with a confocal module (Zeiss LSM 700; Carl Zeiss Microimaging GmbH). Image processing was performed using the ZEN 2009 (Carl Zeiss Microimaging GmbH) and Adobe Photoshop Elements 9 (Adobe Systems Incorporated, San Jose, CA) software.

**Scratch wound and cell proliferation assays** - To analyze the effects of the nucleotides on keratinocyte proliferation, HaCaT cells were seeded on 12-well plates at 80.000 cells/well and grown for 2 days. After this, the cells were treated with 10 µM or 100 µM ATP, AMP or adenosine in medium containing 1% FBS. The cells were trypsinized and counted 24 h later to assess the impact of the treatments as compared to the controls (no added nucleotides).

To analyze differences in migration rates, HaCaT cells were seeded on 24-well plates at 80.000-160.000 cells/well. After 2 days of culture, a cross-shaped area was scraped in each well with a sterile 1 ml pipette tip to get a wound devoid of cells, and fresh medium containing 1% serum with or without 10  $\mu$ M ATP, AMP or adenosine was changed. The control and the nucleotide-treated cells (6-8 parallel wells/treatment) were photographed immediately and 6 h and 24 h after wounding using an Olympus CK2 inverted phase contrast microscope (4x objective) and a Nikon Digital Sight DS-L1 camera system. The distance the cells migrated was analyzed using the ImageJ software (National Institutes of Health, USA; <http://imagej.nih.gov/ij>).

**Statistical methods** - The results were analyzed with PASW Statistics 18 (SPSS Inc., Chicago, IL). Log- or ln-transformation was used if the data was not normally distributed or the variances were unequal, or the non-parametric Friedman test was chosen. Mixed model ANOVA was applied, and pairwise comparisons between the treatments were performed using the estimated marginal means (LSD) or Dunnett's test. The controls (set as 1) and treatments were compared using the cumulative distribution function p-norm in R for Mac OS X, version 3.2.3 [45]. The results were corrected for multiple comparisons. The one-sample t-test was used for comparisons of a single treatment normalized to control (set as 1). Statistical significances are indicated as  $^{*},\#, \$ P < 0.05$ ,  $^{**},\#\#, \$\$ P < 0.01$  and  $^{***},\###, \$\$\$ P < 0.001$ . The data represent means and SEM, unless otherwise indicated.

## RESULTS

### **Extracellular ATP strongly upregulates *HAS2* expression in HaCaT keratinocytes -**

Treating HaCaT with ATP for 2 h strongly upregulated *HAS2* expression (Fig. 1A) in a dose-dependent fashion (Fig. 1B). As the response with 100  $\mu$ M was robust and slightly larger than with 10  $\mu$ M, the higher concentration was used in most subsequent experiments with ATP and its metabolites. Maximum induction occurred at 1.5 h, and subsided to a 50% inhibition at 6 h (Fig. 1C). Another stimulation was seen at 24 h (Fig. 1C).

There were no significant changes in the expression of *HAS1*, *HYAL1-2* or *CD44* at 2 h (Fig. 1A), but *HAS1* increased later, at 6 h (Fig. 1D). ATP also caused a modest increase in the expression of *HAS3*, starting at 2 h (Fig. 1A), and the transcript remained elevated (Fig. 1E). *CD44* showed a pattern similar to that of *HAS3*, being significantly upregulated at 4-6 h (Fig. 1F).

### **Metabolites of ATP differentially regulate *HAS* expression -**

Like ATP, ADP (100  $\mu$ M) strongly upregulated *HAS2* mRNA expression (Fig. 2A). In contrast, the further dephosphorylated AMP downregulated *HAS2* at 2 h (range: 0.27-0.85;  $n = 12$ ,  $P = 0.0001$ ). This inhibitory effect persisted for at least 6 h, but after 24 h *HAS2* expression in the AMP-treated cells exceeded the controls (Fig. 2B). AMP induced a strong expression of *HAS1*, which peaked at 4-6 h (Fig. 2C), while the effects on *HAS3* were more modest, with the transcript levels rising steadily towards 24 h (Fig. 2D).

Resembling the effects of AMP, adenosine inhibited *HAS2* at 2-6 h, whereas the transcripts were restored to control levels by 24 h (Fig. 2B). The effect of adenosine on *HAS1* expression was similar to AMP (Fig. 2C), while it did not significantly affect *HAS3* (Fig. 2D). *CD44* did not exhibit significant responses to either AMP or adenosine (Fig. 2E).

### **ATP upregulates *HAS2* expression predominantly via P2Y<sub>2</sub>, Ca<sup>2+</sup>, and MAPK signalling -**

To test the involvement of the P2Y receptors in the *HAS2* response, siRNAs and chemical inhibitors were applied. The efficiency of the P2Y<sub>2</sub> siRNA was 60% (Fig. 3A), which was associated with a 68% reduction of the ATP-induced *HAS2* upregulation (Fig. 3A). This suggests that P2Y<sub>2</sub> was the

dominant P2Y receptor in the *HAS2*-response, an idea strengthened by the negligible effect of the P2Y<sub>11</sub> inhibitor NF340 (Suppl. Fig. 1A).

Next, ADP receptors were blocked to study whether ATP signals through them. MRS2179, which inhibits P2Y<sub>1</sub>, slightly suppressed the *HAS2*-response (Fig. 3B). Inhibition of P2Y<sub>12</sub> and P2Y<sub>13</sub> with PTX, which releases the G<sub>i</sub> protein from the receptors [46] and effectively blocks G<sub>i</sub>-signaling in HaCaT without exhibiting toxicity [38], moderately inhibited the ATP-induced *HAS2* upregulation (Fig. 3C). Additionally, upregulation of *HAS2* at 2 h by the unhydrolyzable ATP-analog ATP $\gamma$ S was not significantly different from the ATP-induced response (Fig. 3D). This suggests that the metabolites of ATP such as ADP are not required for the upregulation of *HAS2* expression, and further supports the conclusion that P2Y<sub>2</sub> is the main receptor mediating the effect.

Adenosine, most probably acting via the P1 receptors [25], showed a tendency to inhibit the ATP-response (Fig. 3D). Additionally,  $\beta,\gamma$ -methylene ATP (AMP-PCP), an agonist of certain P2X receptors and an efficient local stimulator of cAMP formation when converted to AMP and adenosine [47], had a minor suppressive influence on *HAS2* transcription (Fig. 3D).

Signal transduction from the G<sub>q/11</sub> protein-coupled P2Y<sub>2</sub> involves the secondary messengers diacylglycerol and Ca<sup>2+</sup>, and subsequent activation of downstream kinases including CaMKII [19], MEK/ERK [48], CREB [49] and PKC [50]. Inhibiting CREB activity with two structurally similar chemicals preventing its binding to CBP [51] attenuated the induction in *HAS2* transcription (Fig. 3E). Upstream, inhibitors of CaMKII (KN93) and PKC (BIM; bisindolylmaleimide I) almost totally abolished the ATP-response (Fig. 3F and 3G). The MAPK pathway (MEK/ERK and p38) inhibitors PD98059 and BIRB796 also significantly suppressed the ATP-induced *HAS2* upregulation (Fig. 3H), while the JAK2/EGFR inhibitor AG490 was ineffective (Suppl. Fig. 1B).

Western blotting was used to further characterize the effects of ATP: phosphorylations of p38, ERK, CREB and ATF-1 (another cAMP-dependent transcription factor) were clear and fast with maximum induction after 15-30 min (Fig. 4A-C), while that of pSTAT3-Ser<sup>727</sup> was weaker, and pSTAT3-Tyr<sup>705</sup> appeared later, after 60-120 min (Suppl. Fig. 2A). However, this activation of



STAT3 was apparently not involved in the regulation of *HAS2* expression, as its specific inhibitor STAT IX failed to counteract the ATP-induced increase in *HAS2* mRNA (Suppl. Fig. 2B).

**ATP and AMP/adenosine have opposing effects on the hyaluronan content of human keratinocyte cultures** - The influence of adenosine nucleotides on the hyaluronan content of HaCaT cultures was analyzed with histochemical and biochemical assays. The histochemical stainings showed a clear increase of hyaluronan in the cell layer already 2 h after the application of 100  $\mu$ M ATP (Fig. 5A-B). Hyaluronan was mainly seen at the plasma membranes, colocalized (yellow color) with its receptor CD44, although an increase in the intracellular pool of HA was also evident (Fig. 5D-E, D'-E'; 4 h after adding ATP, white arrowheads in 5E).

For the biochemical analyses, cells were cultured using medium containing 1% FBS, since medium supplemented with 10% FBS alone stimulated hyaluronan synthesis submaximally and almost overwhelmed the increase induced by ATP (data not shown). 4 h after adding 100  $\mu$ M ATP the pericellular hyaluronan was augmented by 32%. After 6 h the peri- and extracellular hyaluronan pools were increased by 67% and 86%, respectively (Fig. 6A-B). At 24 h, there were no differences in either compartment. When the cultures were supplemented with 1 mM glucosamine to increase UDP-GlcNAc, a key substrate and activator of *HAS2*, hyaluronan content was increased even further both in the trypsinase and the medium (total increases with ATP and glucosamine: 216% and 173% after 6 h, respectively; Fig. 6C). Glucosamine alone increased hyaluronan accumulation in the medium only by 37% ( $P < 0.05$ ) and in the trypsinase by 43% (NS), indicating that both treatments are needed for a maximal effect.

Marked changes in hyaluronan stainings were not recognized after 2-4 h treatments with AMP and adenosine (Fig. 5C, 5F and 5F'). The inhibitory effect of AMP on *HAS2* expression was not reflected in hyaluronan accumulation during a 6-h follow-up, while after 24 h a clear 41% reduction was observed in the medium (Fig. 6D). Adenosine caused a significant 38% reduction in pericellular hyaluronan already at 6 h, and also suppressed hyaluronan content in the medium at 24 h by 36% (Fig. 6E).

As intracellular hyaluronan precursor sugars influence HAS-activity (Fig. 6C) [52-54], we measured their concentrations with HPLC. UDP-GlcNAc was consistently decreased after 3-4.5 h in the ATP-treated cultures, whereas the UDP-glucuronic acid (UDP-GlcUA) pool was unaffected (Fig. 6F).

**ATP influences keratinocyte migration** - Since increased hyaluronan synthesis and *HAS2* expression [55,56] have been connected to enhanced cell migration and proliferation, we tested the effects of ATP on keratinocyte numbers and migratory activity in scratch wound assays. 10 and 100  $\mu$ M ATP did not significantly affect cell numbers after a 24-h treatment (Fig. 6G), although the higher dose has been considered potentially growth-inhibiting [23]. In contrast, adenosine significantly lowered, and AMP had a tendency to suppress cell proliferation after a 24-h follow-up period with the 100  $\mu$ M dose (Fig. 6G). We then used for the migration assays the lower (10  $\mu$ M) concentrations, which had no effect on proliferation. Treatment of the cells with this dose of ATP increased their motility by about 23% after a 6-h treatment (Fig. 6H). However, by 24 h this effect had subsided (Fig. 6H). Although adenosine showed a tendency to slow the early phase of scratch wound closure in 3 out of 4 experiments, the effect was not statistically significant (Fig. 6H; after 6 h). After 24 h, no difference, as compared to the untreated cultures, were seen with either adenosine or AMP (Fig. 6H, after 24 h).

Overall, our data indicate that extracellular ATP and its degradation products are potent regulators of keratinocyte *HAS* expression and hyaluronan synthesis as well as cell behavior, although with partially opposing effects. The signaling pathways most likely involved in the changed *HAS* expression are summarized in Figure 7.

## DISCUSSION

The present work shows for the first time that extracellular adenosine nucleotides are potent regulators of *HAS* expression. Importantly, ATP and the less phosphorylated AMP and adenosine often showed opposite influences on *HAS* expression, hyaluronan synthesis and keratinocyte functions, suggesting the existence of a regulatory loop based on ATP catabolism.

***HAS* expression and hyaluronan synthesis** - ATP caused a rapid and very strong *HAS2* upregulation, which was followed by a decline 4-6 h after the ATP exposure, concomitantly with increased *HAS1* and *HAS3* expressions. AMP and adenosine also upregulated *HAS1* and *HAS3* transcript levels; however, their influence on *HAS2* was inhibitory. As hydrolysis of extracellular ATP is rapid in HaCaT [14], the downregulation of *HAS2* and the rise of *HAS1* and *HAS3* 4-6 h after the ATP exposure may be caused by these degradation products.

ATP exposure was associated with a rapid increase in pericellular hyaluronan content and in the amount of hyaluronan released to the culture medium, in line with the observed changes in *HAS* mRNA expressions. However, the fold change in *HAS2* mRNA with ATP was very high, actually higher than previously seen with potent stimuli such as growth factors or hormones [57-60], and compared to the *HAS2* response the change in the hyaluronan content was relatively modest, suggesting that ATP may have caused other, perhaps posttranslational changes in *HAS2* activity. It is also possible that the changes in the mRNA and protein levels do not correspond to each other under our experimental conditions, or that the residence and activity of the *HAS2* protein at the plasma membrane does not reflect the true extent of the changes in its expression [54].

Indeed, we observed that ATP reduced the level of UDP-GlcNAc after ~ 4 h. This could decrease hyaluronan synthesis through a lower precursor supply and by inhibiting the enzymatic activity due to reduced *O*-GlcNAcylation of *HAS2* [61]. Extracellular ATP as well as adenosine can also signal for AMPK activation [62], which can inhibit both the activity of *HAS2* [63] and of GFAT1, the key enzyme in the hexosamine pathway towards UDP-GlcNAc [53,64]. Indeed, supplementation of the cultures with glucosamine to enlarge the cellular pool of UDP-GlcNAc

[52,65] significantly augmented hyaluronan synthesis in the ATP-treated cultures, suggesting that reduced precursor supply dampened the hyaluronan response with the ATP treatment.

Subjecting keratinocytes to either AMP or adenosine reduced the content of hyaluronan, consistently with their inhibitory effect on *HAS2* expression, despite the simultaneous upregulation of the *HAS1* isoform. However, in this case the fold changes may be deceptive, as the basal expression level of *HAS1* is very low in keratinocytes, and the synthetic activity of this isoenzyme is modest [29,65].

**Receptors and signals of extracellular adenine nucleotides** - The concentrations of extracellular nucleotides are generally considered to be nano- or (sub)micromolar in quiescent cells and normal tissues [25,66,67], being thus considerably lower than those used here. However, extracellular ATP concentrations close to what we used in the present experiments (10 and 100  $\mu\text{M}$ ) are found in stimulated polymorphonuclear leukocytes [68], irritated keratinocytes [69] and wounded epithelial cells [10]. Even higher concentrations of ATP occur under physio-pathological stress such as contact dermatitis and tumor microenvironments [70]. Hypoxia increases extracellular adenosine levels: in the extracellular fluid of hypoxic brain [71] and in solid tumors [72] adenosine can reach micromolar concentrations.

The release of ATP after various insults often seems to be an immediate response, but may also depend on the trigger. Thus, after UVB [7] and UVA irradiation [73] as well as stretching [74] the release occurs within seconds/minutes. After chemical irritation the release has been reported to occur within one hour, while after non-metal hapten exposure a longer period is required [75].

The degradation of ATP in HaCaT cell cultures has been reported to occur rapidly reaching a stable level of approximately 50% during the first 40 min after the change of fresh medium (endogenous ATP), while a decline to ~ 60% has been found 1 h after exogenously added ATP [14]. Deduced from this more than half of the ATP is probably degraded within the 4-hour time frame, when the inhibitory effect on *HAS2* and the stimulatory effect on *HAS1* after ATP was observed, allowing time for signaling of the less phosphorylated derivatives.

Considering the signaling pathways, keratinocytes are known to express multiple purinergic receptors both from the P2Y and P2X families [7,11,20]. Experiments with siRNAs and chemical inhibitors indicated that P2Y<sub>2</sub> is the principal player in the ATP-induced *HAS2* response, while P2Y<sub>1</sub> and P2Y<sub>12/13</sub> play minor roles. However, we cannot totally exclude contributions from the P2X-family, also expressed in epidermal keratinocytes.

AMP does not have unique receptors, but it could use A<sub>1</sub>, one of the receptors for adenosine [76]. However, inhibition of A<sub>1</sub> failed to prevent the AMP-induced *HAS2* downregulation (unpublished observations), in line with its low expression in keratinocytes [22]. Therefore, it is likely that AMP influences *HAS* expression via conversion to adenosine.

At least under normal conditions [77] the main adenosine receptor in keratinocytes is A<sub>2B</sub> [21,22], the activation of which inhibits keratinocyte proliferation and causes cell cycle arrest through increased intracellular calcium [77]. A<sub>2A</sub>, another adenosine receptor, little expressed in resting keratinocytes, is upregulated in psoriatic epidermis [77], and by cytokines such as Il-1 $\beta$  and TNF $\alpha$ , indicating that adenosine signaling shifts to this receptor under inflammatory conditions. This leads to cell proliferation via p38 signaling [77]. Since adenosine reduced cell numbers, the expression of the adenosine receptor A<sub>2B</sub> rather than A<sub>2A</sub> prevailed in the present experiments. Intriguingly, if the expression of A<sub>2A</sub> dominates, which can occur in stressed tissues [77] or malignancies [78], activation of p38 signaling could increase the expression of *HAS2*, as observed in keratinocytes [35,39], and actually lead to increased hyaluronan production.

However, adenosine can also signal via receptor-independent mechanisms such as through adenosine uptake, followed by conversion to AMP with its intracellular signaling functions [79], or by influencing membrane phosphatase activity [77]. The delayed responses, including the upregulation of *HAS1* after 4-6 h and the secondary waves of *HAS2* and *HAS3* activation after 24 h, are likely processes secondary to the initial signals induced by the nucleotides. Interestingly, the induction seen in *HAS1* with adenosine parallels the observations of Grandoch et al. [37] in human coronary artery smooth muscle cells, where *HAS1* was most strongly upregulated after a 6-h

treatment with the adenosine analog 5'-N-ethylcarboxamidoadenosine (NECA).

Extracellular ATP inherently induces  $\text{Ca}^{2+}$  release and PKC signaling via the  $\text{G}_{q/11}$ -coupled P2Y-receptors [18], activating MAP kinases, CaMKII and CREB. The upregulation of *HAS2* by CREB is anticipated since it has functional binding sites at the proximal *HAS2* promoter [80]. Additionally, CaMKII, p38 and ERK regulate *HAS* expression in response to radiation stress and growth factors [35,55,81,82], and p38 and ERK respond to extracellular ATP in human keratinocytes [83,84].

The time frame of the phosphorylations in p38, ERK and CREB, with maxima 15-30 min after starting the treatments, coincides well with the maximum induction seen in *HAS2* around 90-120 min. Despite the functional STAT3 response element on the *HAS2* promoter [85], and distinct STAT3 phosphorylation at two sites after the ATP exposure, experiments with the STAT3 inhibitor indicated that *HAS2* is not directly induced by STAT3. The modest increase in the phosphorylation level of Ser<sup>727</sup> may not have been enough to activate *HAS2* transcription. The more delayed Tyr<sup>705</sup> phosphorylation suggests a separate signaling route. In line, our preliminary experiments suggested that AMP also induced STAT3 phosphorylation at Tyr<sup>705</sup> but not at Ser<sup>727</sup> (data not shown). Such a difference in STAT3 phosphorylations may significantly influence the final outcome [86].

Extracellular UTP, also released from stressed cells, causes an upregulation of *HAS2* similar to that induced by ATP [39]. This is not surprising since they share P2Y<sub>2</sub>, the main P2 receptor involved in the *HAS2* response. UTP and ATP also have many common signaling steps involved in the upregulation of *HAS2*, including MAP kinases, CaMKII and CREB [39]. However, ATP and UTP have also divergent effects on signaling, most obviously in STAT3 activation and its involvement in *HAS2* upregulation [39]. This is probably explained by the contribution of receptors specific for each nucleotide, including P2Y<sub>4</sub> for UTP, P2Y<sub>1</sub> and P2Y<sub>12/13</sub> for ADP, and P2X for ATP. The difference in the signaling between ATP and UTP is signified by their divergent effects on *HAS1* and *HAS3* transcription, and the ability of AMP, but not UMP, to reduce *HAS2* expression [39].

**Keratinocyte proliferation and migration** - In addition to acting in the maintenance of extracellular matrices, *HAS* expression and hyaluronan content have been reported to regulate cell functions including proliferation, migration, differentiation and apoptosis [87]. The inhibition of hyaluronan synthesis induced by adenosine in HaCaT cells was associated with a reduction in cell numbers, in line with previous reports on human keratinocytes [21,77]. Here, ATP did not clearly affect cell proliferation, which appears to depend on the experimental setup [20,23,88].

However, ATP stimulated keratinocyte migration, and this coincided with the accumulation of hyaluronan after a 6-h ATP treatment. Such stimulation might be enough to keep keratinocytes activated and even repair minor epidermal damages, however, other factors are needed to mount a full wound healing response [89]. Although exogenous ATP has been shown to stimulate cell migration in many cell types such as in corneal epithelial cells [90], its influence on keratinocyte motility has been controversial [91], perhaps due to differences in the experimental conditions used. Endogenously released ATP is more consistently stimulatory, regulating stretch-induced and basal migration [74], as well as enhancing epithelial cell motility and wound closure [92].

There is no previous information about the influence of adenosine on keratinocyte migration. However, it stimulates motility in inflammatory cells and in breast cancer cells via autocrine activation of the  $A_3$  receptor, but inhibits cell migration when added exogenously ([93], and references therein). The expression of this receptor is low in keratinocytes [77], perhaps explaining why no change in migration was found. Actually, there was a tendency towards reduced migration at the early phase, coincidentally with the inhibition of *HAS2* expression. However, adenosine may still play a positive role in skin wound healing since NECA, its nonspecific analog, has been shown to counteract corticosteroid-suppressed wound healing in a mouse model [94].

**Extracellular nucleotides and hyaluronan in epidermal biology** - Extracellular nucleotides are potent local danger signals that affect skin homeostasis through altered gene expression and matrix remodeling, as previously demonstrated in dermal fibroblasts during wound healing [95]. Especially signaling via the  $P2Y_2$ -receptor is implicated. The present data show that extracellular

nucleotides regulate the synthesis of hyaluronan, a major component of the ECM in the epidermis [32], through the same receptor.

During normal tissue homeostasis hyaluronan synthesis and degradation in the epidermis are active, but balanced [96]. However, whenever this homeostasis is disturbed with insults such as wounding, barrier disruption or UVB radiation, epidermal hyaluronan content is rapidly increased [33-35], even within the first hour after the trauma [97], together with other immediate defence reactions in the tissue. The strong but transient surge of *HAS2* expression is obviously intended for a rapid response that can be augmented by other activators such as growth factors, hormones or cytokines, if the noxious condition prevails [98-100]. However, extracellular nucleotides may also play a role in the prolonged hyaluronan response because of the more widespread and/or increased expression of the P2Y<sub>2</sub> receptor induced by e.g. wounding and UVB [101,102].

As changes in *HAS*-expression and protein kinase activation elicited by UVB resemble those caused by the ATP-exposure [35,103], they may indeed be related to nucleotide signaling [8]. However, while we have observed that the initial influences of UVB on rat keratinocyte *Has2* and *Has1* [35] resemble those of adenosine on HaCaT, UVB-exposure causes *Has2* to peak bimodally at 8 h and 36 h in a p38-dependent manner, perhaps due to a secondary release of ATP induced by the initial surge of extracellular nucleotides [104] or altered expression of the P2Y<sub>2</sub> receptor [102,105,106].

Functionally, the hyaluronan accumulation seen after ATP-treatment may protect cells from apoptosis, as shown in UV-stressed dermal fibroblasts [107] and corneal epithelial cells [108]. Hyaluronan acting together with its receptor CD44 supports cell survival via AKT signaling, and CD44 may prevent apoptosis via counteracting ROS activity (reviewed in [109]). Interestingly, the increased expression of CD44 seen after epidermal insults closely correlates with hyaluronan accumulation [33], and this pattern was also seen here after the ATP exposure.

**Conclusions** – Our data showing the transient nature of the ATP-induced *HAS* upregulation is physiologically important, constituting a “state of alert”, which can be either amplified by other



factors, or shut down by the ATP degradation products AMP and adenosine in the absence of additional signals for tissue damage. On the other hand, adenosine under basal conditions tends to subdue hyaluronan production to maintain epidermal tissue homeostasis.

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**DECLARATIONS OF INTEREST**

The authors state no conflict of interest.

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**AUTHOR CONTRIBUTION STATEMENT**

LR planned and performed part of the experiments, analyzed data, contributed to paper writing and prepared the figures. TJ conceived the study, analyzed data and contributed to paper writing. RHT coordinated the study, analyzed results and participated in the writing of the paper. RK provided technical expertise for most of the experiments. GB helped conceptualize the study, present the data and revise the text. SO, PT and SPS performed and analyzed part of the experiments, and critically revised the manuscript. MIT took part in the study conception and critically revised the manuscript. All authors approved the manuscript.

**SUPPLEMENTARY MATERIAL**

Refer to the online version for Supplementary Figures 1-2 and Supplementary Table S1.

**FOOTNOTES**

<sup>a</sup>An earlier version of this manuscript has been included in the doctoral thesis work of Leena Rauhala, PhD, and published (print only) in the Publications of the University of Eastern Finland – Dissertations in Health Sciences (2017).

<sup>b</sup>Abbreviations used:

CaMKII = Ca<sup>2+</sup>/calmodulin-dependent protein kinase II

CREB = calcium response element binding protein

HA-ELSA = hyaluronan enzyme-linked sorbent assay

HAS = hyaluronan synthase

MAPK = mitogen-activated protein kinase

P1, P2X, P2Y = purinergic (nucleotide and nucleoside) receptors

PKC = protein kinase C

STAT = signal transducer and activator of transcription

UDP-GlcNAc = uridine diphosphate N-acetyl glucosamine

UVB = ultraviolet B radiation

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## FIGURE LEGENDS

**Figure 1. Extracellular ATP upregulates *HAS* expression in HaCaT keratinocytes.** (A) The expression levels of *HAS1* ( $n = 4$ ), *HAS2* ( $n = 8$ ), *HAS3* ( $n = 4$ ), *HYAL1* and *HYAL2* ( $n = 3$ ) and *CD44* ( $n = 3$ ). (B) *HAS2* dose response ( $n = 2$ ). Time course of the expression of (C) *HAS2* ( $n = 3$ ), (D) *HAS1* ( $n = 3$ ), (E) *HAS3* ( $n = 3$ ) and (F) *CD44* ( $n = 3$ ). Means and ranges are shown in (B); means and SEM in the other panels. The significance of the effects of ATP was analyzed in (A) with one-sample t-test, in (B-C, E) with Friedman test, and in (D, F) with mixed model ANOVA and the pnorm-function. In E, the individual time points were further analyzed using a single group t-test comparing to 1. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

**Figure 2. The degradation products of ATP differentially regulate *HAS* expression.** HaCaT cells were treated with 100  $\mu\text{M}$  ADP, AMP or adenosine. (A) The effect of ADP on *HAS2* ( $n = 4$ ). (B-E) Time course of the effects of AMP and adenosine on *HAS1*, *HAS2*, *HAS3*, and *CD44* ( $n = 3$  for each). The data (means and SEM) were analyzed in (A) with one-sample t-test and in (B-E) with mixed model ANOVA and the pnorm-function. Significances indicate pairwise comparisons to the controls, set as 1. \*<sup>#</sup>  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*<sup>###</sup>  $P < 0.001$ .

**Figure 3. Upregulation of *HAS2* expression by ATP involves calcium and MAPK signaling via *P2Y2*.** HaCaTs subjected to various inhibitors and *P2Y2* siRNA were treated with ATP and adenosine analogs for 2 h, and analyzed for *HAS2* mRNA. (A) Treatment with control or *P2Y2* siRNAs, (B) the *P2Y1*-inhibitor MRS2179 (5  $\mu\text{M}$ ), (C) the  $G_i$ -inhibitor pertussis toxin (PTX), (D) ATP, ATP + adenosine, ATP $\gamma$ S and  $\beta\gamma$ -meATP, (E) the CREB antagonizing naphthol AS-E phosphate (AS-E) and naphthol AS-BI phosphate (AS-BI), (F) the CaMKII-inhibitor KN93, (G) the PKC-inhibitor BIM, and (H) the MEK-inhibitor PD98059 and the p38-inhibitor BIRB796 (means and SEM). The data were analyzed using mixed model ANOVA and the pnorm-function, or (F) Friedman and Wilcoxon tests. \*  $P < 0.05$ , \*\*<sup>##</sup>  $P < 0.01$ , \*\*\*<sup>###,§§§</sup>  $P < 0.001$ .

**Figure 4. ATP induces the phosphorylation of p38, ERK and CREB.** HaCaTs were treated with ATP for 15-120 min before collecting total cell lysates. The phosphorylation status of (A) p38, (B) ERK1/2 and (C) CREB was analyzed by western blotting. Quantifications of the specific protein bands normalized to actin (means and SEM;  $n = 3$ ) are shown with representative blots. For pERK, the 42 and 44 kDa bands were calculated together. pATF-1 also recognized by the anti-pCREB-S133 was not quantified. Mixed model ANOVA or Friedman test (in B) was used to analyze the effect of ATP in the time series. \*  $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

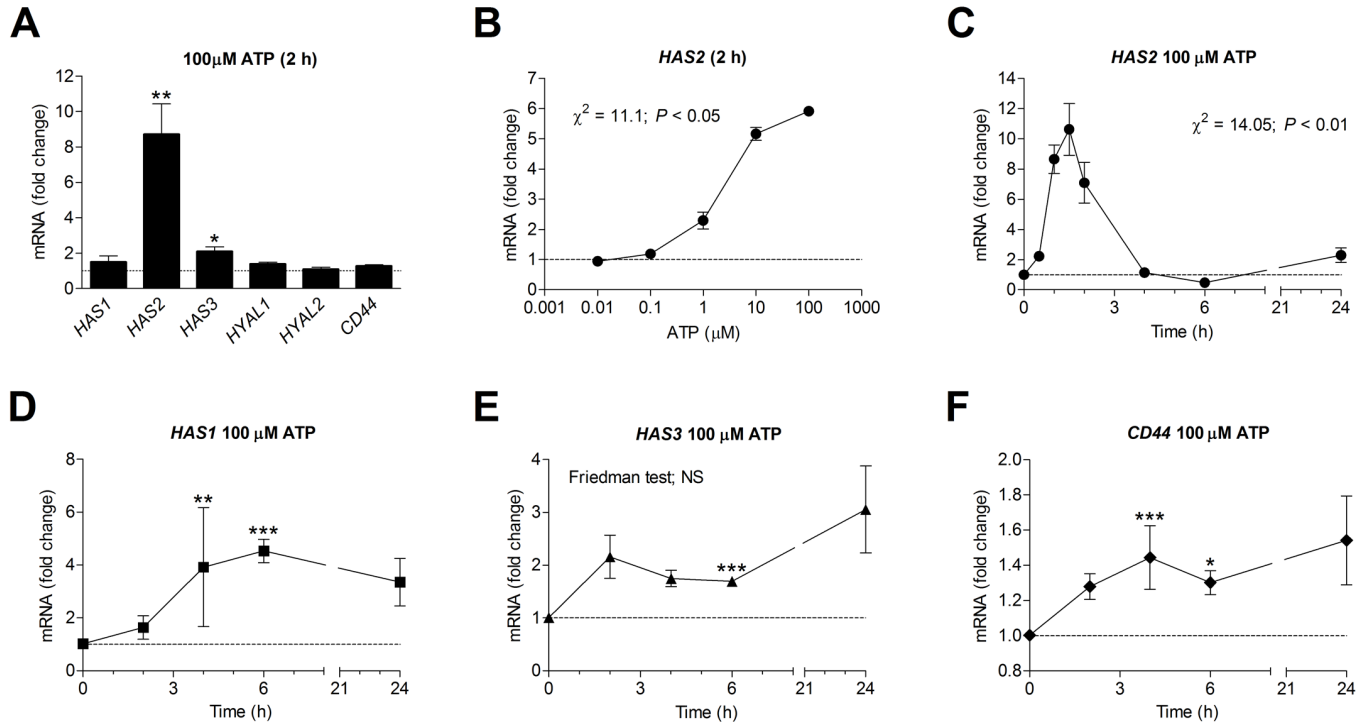
**Figure 5. ATP induces pericellular accumulation of hyaluronan.** HaCaTs were treated with 100  $\mu\text{M}$  of ATP, AMP or adenosine. (A-C) The cultures were stained for hyaluronan using DAB as a chromogen after a 2-h treatment. (D-F) Double stainings for hyaluronan (HA; red) and CD44 (green) were performed using a biotinylated hyaluronan binding probe and TR-labeled streptavidin for hyaluronan, and FITC-labeled anti-mouse IgG antibody for CD44 after a 4-h treatment. The confocal images in (D-F) represent compressed stacks of optical sections and those in (D'-F') their (vertical) side views. The nuclei were stained with hematoxylin in (A-C) and with DAPI (blue) in (D-F). The white arrowheads indicate intracellular hyaluronan. Magnification bars: 100  $\mu\text{m}$  in (A-C), and 20  $\mu\text{m}$  in (D-F).

**Figure 6. Extracellular adenosine nucleotides differentially regulate hyaluronan metabolism.** (A-E) HaCaTs cultured in 1% serum were treated with 100  $\mu\text{M}$  ATP, AMP or adenosine (4-24 h) and 1 mM glucosamine (GlcN, 6 h). Hyaluronan (HA) content was analyzed with the sandwich-type HA-ELSA ( $n = 3$ ). (F) The intracellular nucleotide sugars (UDP-N-acetylglucosamine, UDP-GlcNAc; UDP-glucuronic acid, UDP-GlcUA) in HaCaTs treated with 100  $\mu\text{M}$  ATP ( $n = 3$ ). (G) HaCaT cell numbers analyzed 24 h after adding the nucleotides (10-100  $\mu\text{M}$ ;  $n = 3$  for each). (H) The migration of HaCaT was analyzed 6 h and 24 h after wounding and adding 10  $\mu\text{M}$  ATP ( $n = 6$ ), and 10  $\mu\text{M}$  AMP or adenosine (ADE);  $n = 4$  in each. The data (means and SEM) were analyzed

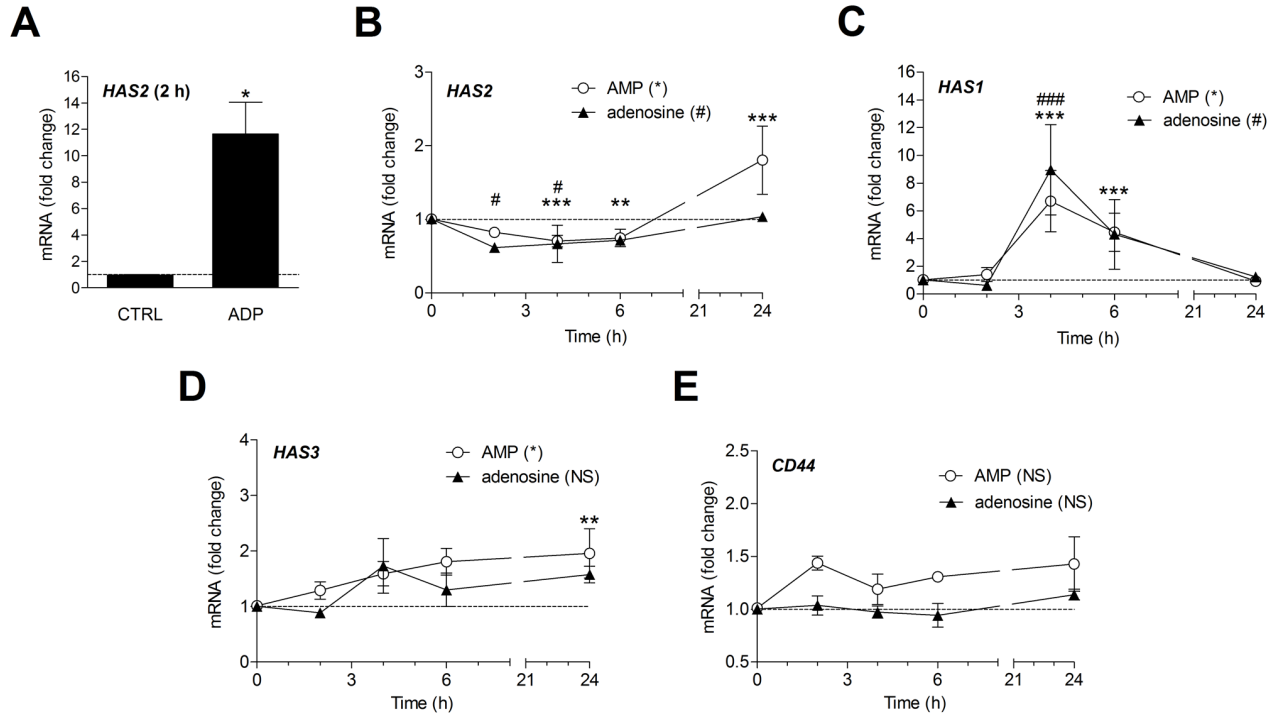
with mixed model ANOVA (**A-E, G-H**) and Friedman test (**F**). \*,#  $P < 0.05$ , \*\*,§§  $P < 0.01$ , \*\*\*,###,§§§  $P < 0.001$ .

**Figure 7. Summary of ATP-induced signaling regulating hyaluronan synthesis in HaCaT keratinocytes.** Summary of the different signaling pathways that are likely to contribute to the increased *HAS2* expression and hyaluronan synthesis in HaCaT keratinocytes. Green ovals and polygons represent those intracellular signaling effectors whose contribution was tested in this study. Solid arrows indicate canonical pathway components, whereas less well-known/alternative connections and interactions are indicated by dashed arrows.

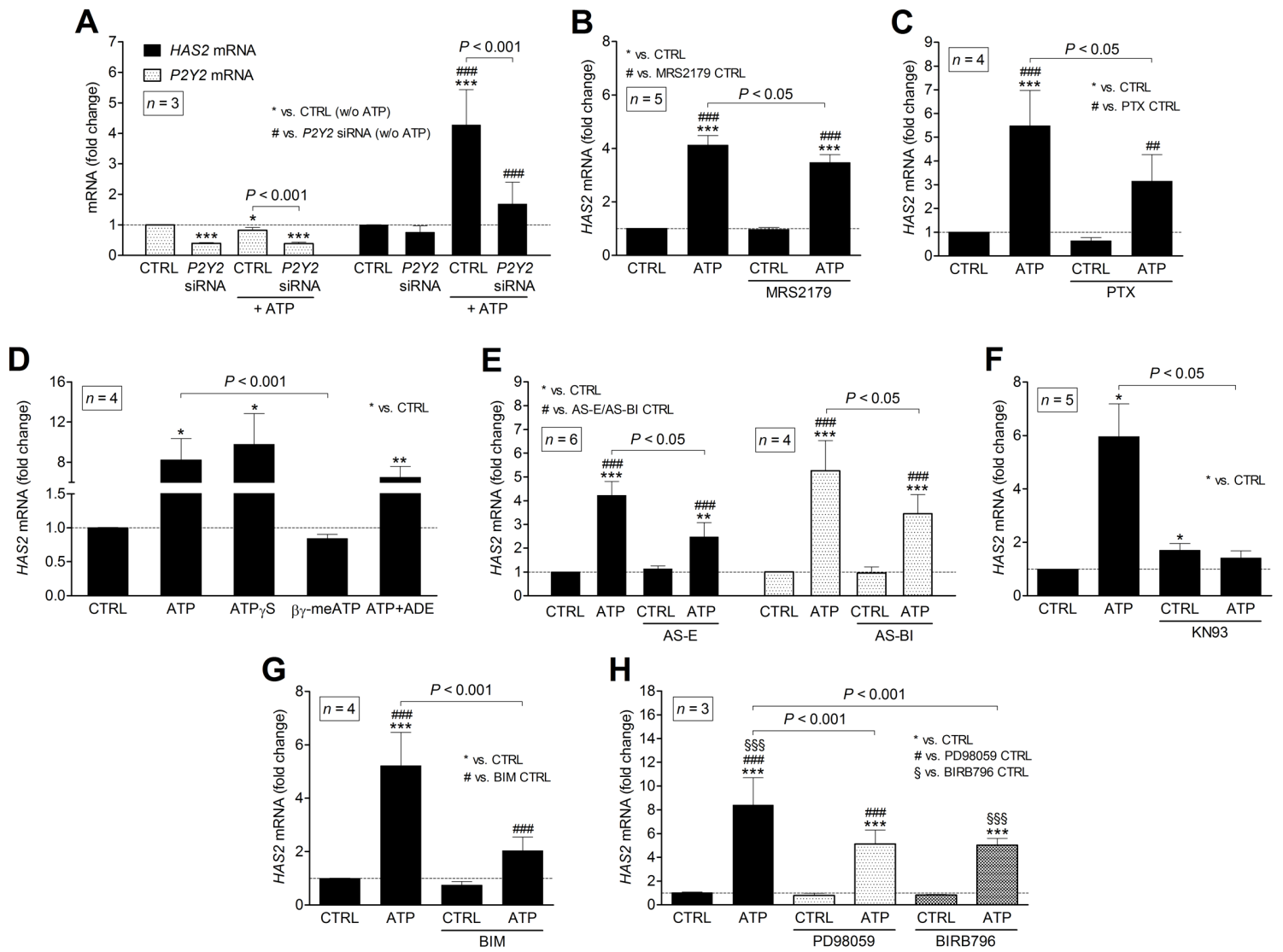
# FIGURE 1



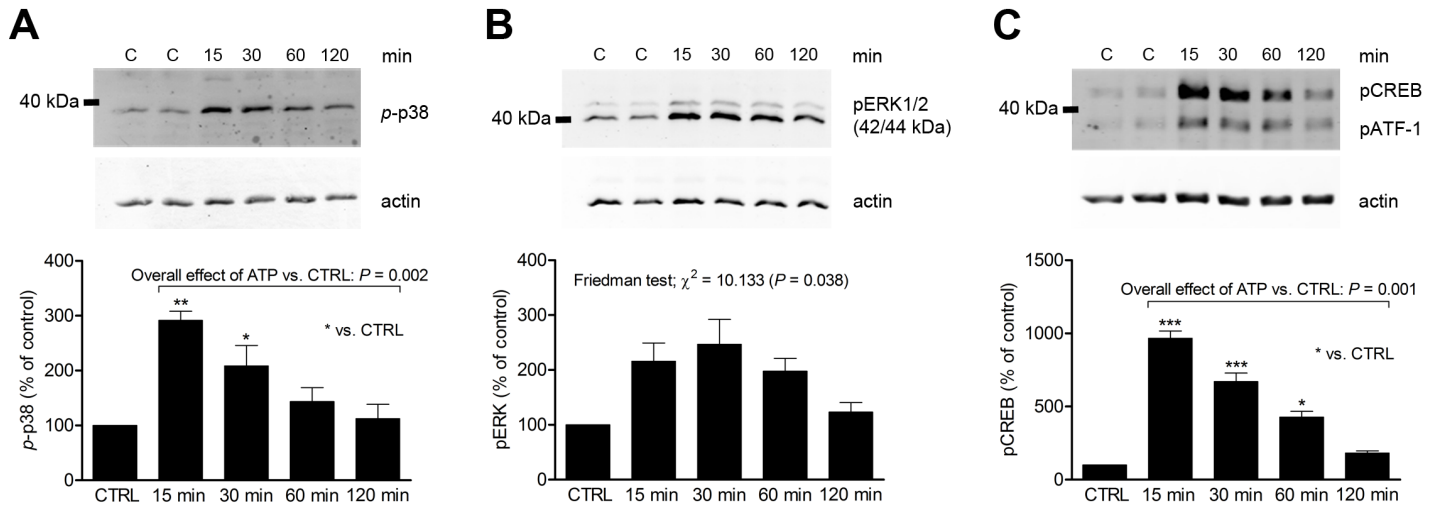
# FIGURE 2



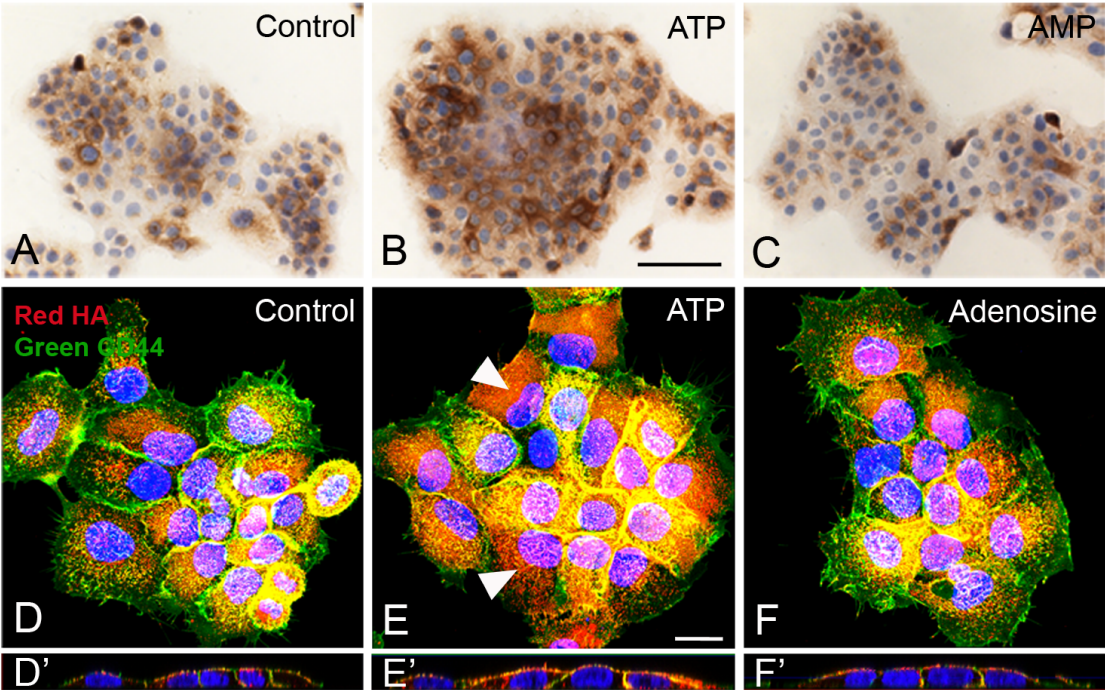
# FIGURE 3



# FIGURE 4

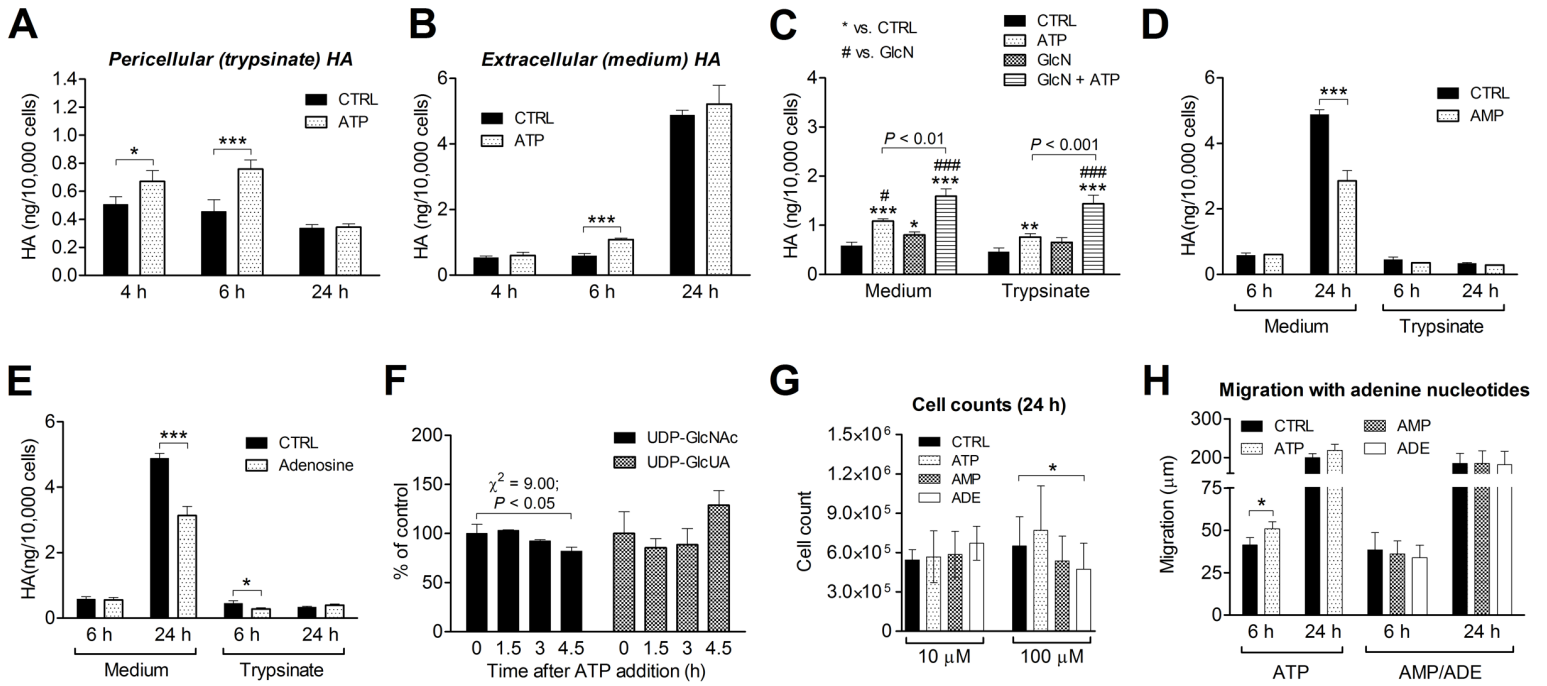


**FIGURE 5**

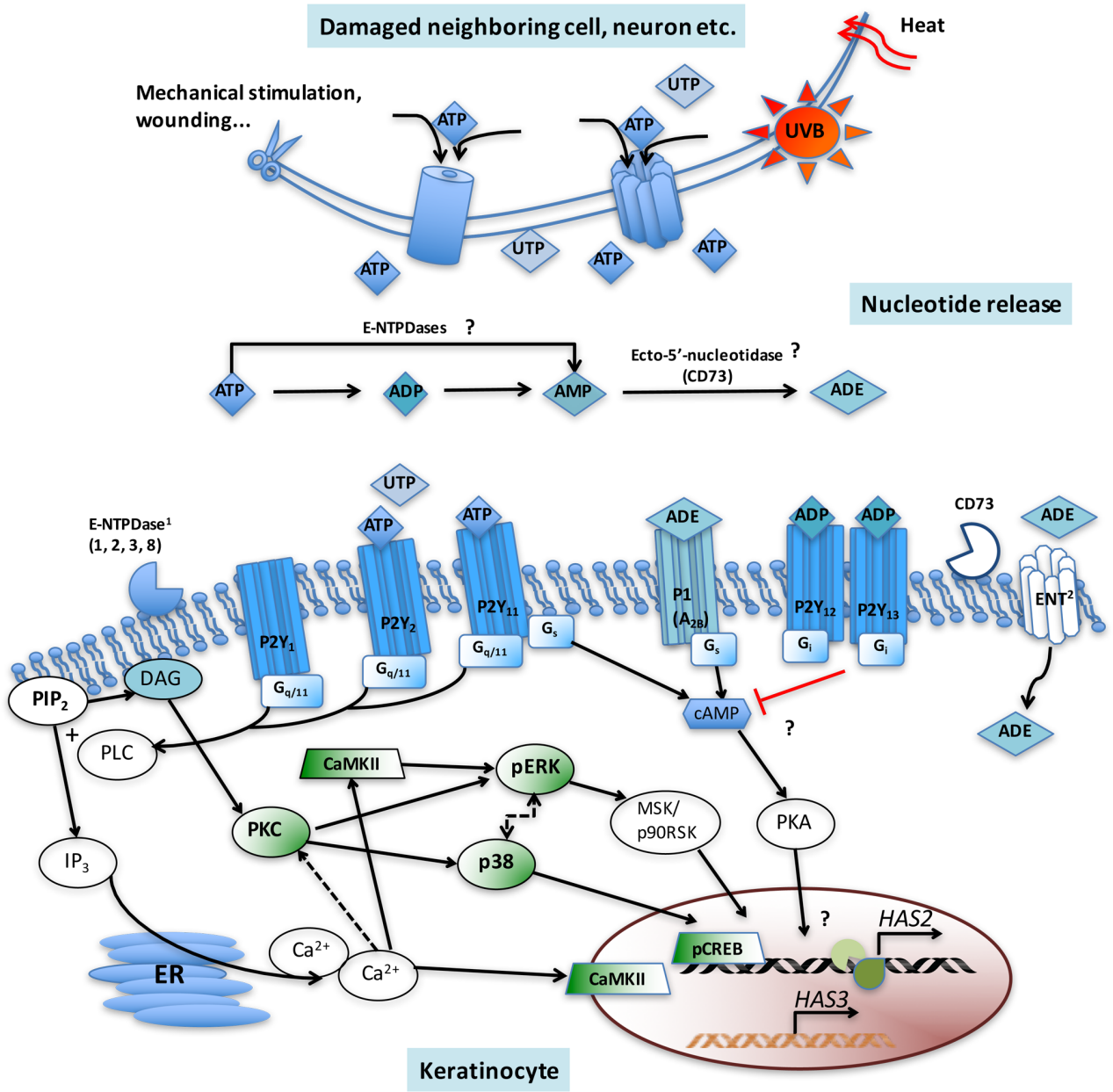




# FIGURE 6



# FIGURE 7



<sup>1</sup>E-NTPDase = Ecto-nucleoside triphosphate diphosphohydrolase  
<sup>2</sup>ENT = equilibrative nucleoside transporter

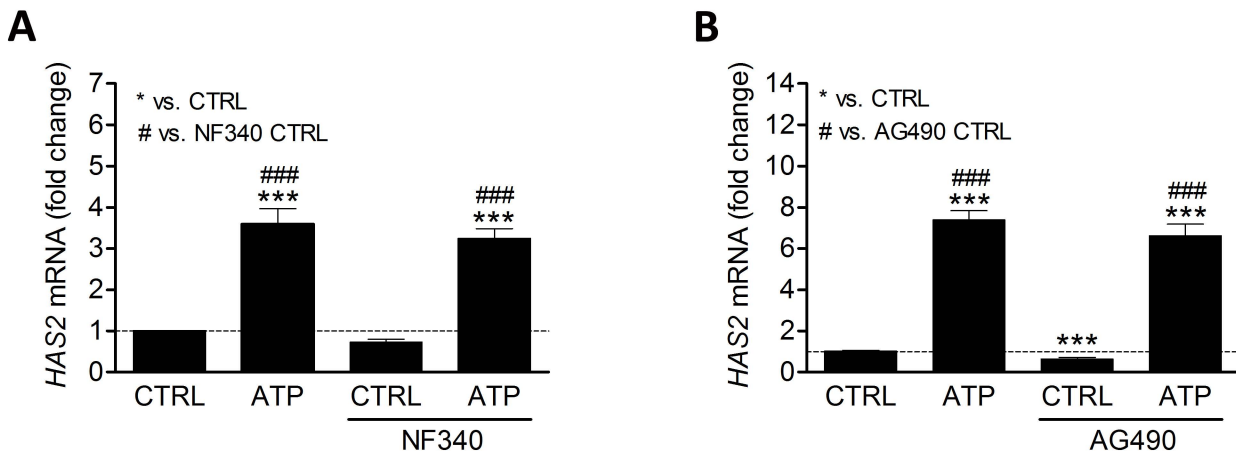
**Supplementary Table S1.** Primer sequences for qRT-PCR of reverse transcribed human genes.

<b>Gene name</b>	<b>Primer sequence (5' to 3')</b>	<b>Annealing temperature</b>
<i>ARPO</i>	Forward: AGATGCAGCAGATCCGCAT Reverse: GTGGTGATACCTAAAGCCTG	59 °C
<i>HAS1</i>	Forward: CAAGATTCTTCAGTCTGGAC Reverse: TAAGAACGAGGAGAAAGCAG	59 °C
<i>HAS2</i>	Forward: CAGAATCCAAACAGACAGTTC Reverse: TAAGGTGTTGTGTGTGACTG	59 °C
<i>HAS3</i>	Forward: CTTAAGGGTTGCTTGCTTGC Reverse: GTTCGTGGGAGATGAAGGAA	59 °C
<i>HYAL1</i>	Forward: GCCCTCTATCCCAGCATCTA Reverse: CTCACCCAGAGCACCCTC	59 °C
<i>HYAL2</i>	Forward: CCTCTGGGGCTTCTACCTCT Reverse: CTGAACACGGAAGCTCACAA	59 °C
<i>CD44</i>	Forward: CATCTACCCCAGCAACCCTA Reverse: CTGTCTGTGCTGTCGGTGAT	59 °C
<i>P2Y2</i>	Forward: CCTGAGAGGAGAAGCGCAG Reverse: GAACTCTGCGGGAAACAGGA	59 °C

## Supplementary Figure 1.

Rauhala, Jokela, Kärnä, Bart, Takabe, Oikari, Tammi, Pasonen-Seppänen and Tammi.

Extracellular ATP activates hyaluronan synthase 2 (*HAS2*) in epidermal keratinocytes via P2Y<sub>2</sub>, Ca<sup>2+</sup>-signaling, and MAPK pathways.

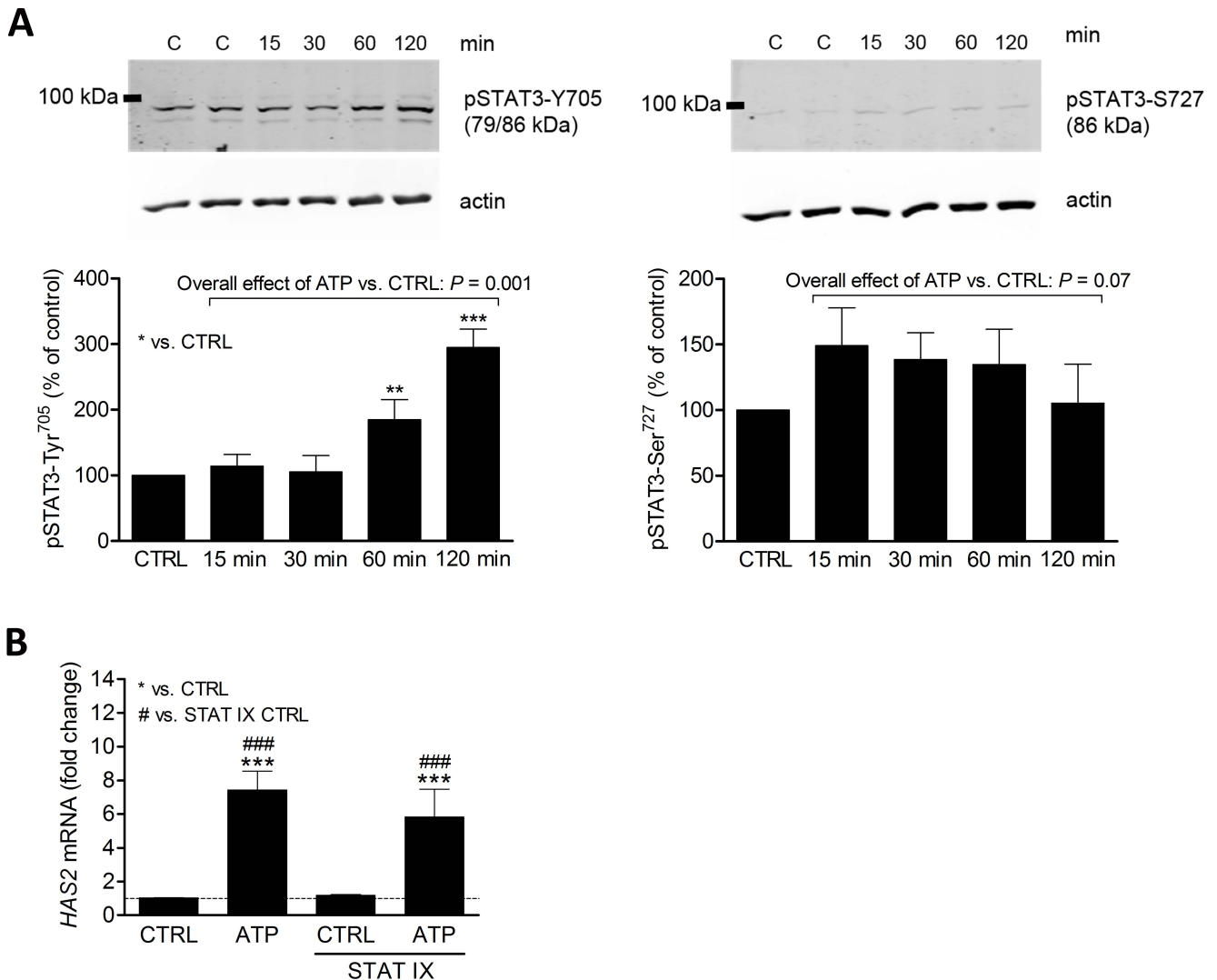


**Supplementary Figure 1. P2Y<sub>11</sub> and JAK2/EGFR do not modulate *HAS2* expression in response to ATP.** HaCaT cells were treated with 100  $\mu$ M ATP for 2 h with or without a pretreatment with chemical inhibitors as described in *Materials and methods*. *HAS2* expression was analyzed in response to **(A)** the P2Y<sub>11</sub>-inhibitor NF340 (n = 3) and **(B)** the JAK2/EGFR inhibitor AG490 (n = 3). Statistical significances of the differences between the treatments were tested using mixed model ANOVA correcting for multiple comparisons in pairwise analyses. The treatments were compared to the controls (set as 1) using the pnorm-function. Statistical significances are indicated as \*\*\*, ###  $p < 0.001$ . The data represent means and SEM.

## Supplementary Figure 2.

Rauhala, Jokela, Kärnä, Bart, Takabe, Oikari, Tammi, Pasonen-Seppänen and Tammi.

Extracellular ATP activates hyaluronan synthase 2 (*HAS2*) in epidermal keratinocytes via P2Y<sub>2</sub>, Ca<sup>2+</sup>-signaling, and MAPK pathways.



**Supplementary Figure 2. STAT3 does not modulate *HAS2* expression in response to ATP despite being activated by the nucleotide treatment.** **A)** HaCaTs were treated with ATP for 15-120 min before collecting total cell lysates. The phosphorylation status of STAT3-Y705 and STAT3-S727 was analyzed by western blotting. STAT3-Y705 showed a clear activation after 60-120 min. Quantifications of the specific protein bands normalized to actin (means and SEM; n = 3) are shown with representative blots. **B)** HaCaT cells were treated with 100  $\mu$ M ATP for 2 h with or without a pretreatment with the STAT3 inhibitor IX (Cpd188) as described in *Materials and methods*. *HAS2* mRNA expression was analyzed by qRT-PCR (n = 5; means and SEM). Mixed model ANOVA was used to analyze the overall effect of ATP in the time series (**A**). Statistical significances of the differences between the treatments were tested using mixed model ANOVA correcting for multiple comparisons in pairwise analyses, and the treatments were compared to the controls (set as 1) using the pnorm-function (**B**). Statistical significances are indicated as \*\* p < 0.01; \*\*\*, ### p < 0.001.