P2Y2 receptor inhibits EGF-induced MAPK pathway to stabilise keratinocyte hemidesmosomes

Emilie Faure[1], Françoise Garrouste[1], Fabrice Parat[1], Sylvie Monferran[2], Ludovic Leloup[1], Gilbert Pommier[1], Hervé Kovacic[1] and Maxime Lehmann[3,*]

[1]Aix-Marseille Université, INSERM UMR 911, Centre de Recherche en Oncologie Biologique et en Oncopharmacologie, Marseille 13005, France
[2]Université Paul Sabatier, INSERM CNRS U1037, Centre de Recherches en Cancérologie de Toulouse, Toulouse 31052, France
[3]CNRS UMR 7213; Université de Strasbourg, Illkirch 67501, France

*Author for correspondence (maxime.lehmann@unistra.fr)

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Summary

α6β4 integrin is the main component of hemidesmosomes (HD) that stably anchor the epithelium to the underlying basement membrane. Epithelial cell migration requires HD remodelling, which can be promoted by epidermal growth factor (EGF). We previously showed that extracellular nucleotides inhibit growth factor-induced keratinocyte migration. Here, we investigate the effect of extracellular nucleotides on α6β4 integrin localisation in HD during EGF-induced cell migration. Using a combination of pharmacological inhibition and gene silencing approaches, we found that UTP activates the P2Y2 purinergic receptor and Gαq protein to inhibit EGF/ERK1/2-induced cell migration in keratinocytes. Using a keratinocyte cell line expressing an inducible form of the Raf kinase, we show that UTP inhibits the EGF-induced ERK1/2 pathway activation downstream of Raf. Moreover, we established that ERK1/2 activation by EGF leads to the mobilisation of α6β4 integrin from HD. Importantly, activation of P2Y2R and Gαq by UTP promotes HD formation and protects these structures from EGF-triggered dissolution as revealed by confocal analysis of the distribution of α6β4 integrin, plectin, BPAG1, BPAG2 and CD151 in keratinocytes. Finally, we demonstrated that the activation of p90RSK, downstream of ERK1/2, is sufficient to promote EGF-mediated HD dismantling and that UTP does not stabilise HD in cells expressing an activated form of p90RSK. Our data underline an unexpected role of P2Y2R and Gαq in the inhibition of the ERK1/2 signalling pathway and in the modulation of hemidesmosome dynamics and keratinocyte migration.

Key words: Purinergic receptor, Hemidesmosome, β4 Integrin, p90RSK, Gαq, MAPK, ERK1/2, Migration, Keratinocyte

Introduction

Hemidesmosomes (HD) are multiprotein complexes that promote stable adhesion of basal epithelial cells to the underlying membrane. In the skin, HD consist of integrin α6β4, type XVII collagen BPAG2 (also called BP180), tetraspanin CD151 and two plakin family members, plectin and BPAG1 (also called BP230). HD formation occurs via the association of the integrin α6β4 and BPAG2 (both binding laminin-332) with keratin intermediate filaments through the plakin proteins plectin and BPAG1 (Litjens et al., 2006; Margadant et al., 2008). HD stability is crucial for tissue integrity and mutations or deletions of any of the HD components result in severe skin blistering diseases (Borradori and Sonnenberg, 1996).

It has been assumed for a long time that HD are stationary molecular complexes that mediates stable adhesion. However, several recent works showed that HD are dynamics structures and that their dismantling and remodelling are involved both in physiological and pathological processes such as keratinocyte migration (notably during wound healing) or carcinoma progression and invasion (Mariotti et al., 2001; Litjens et al., 2006; Mercurio et al., 2001; Guo and Giancotti, 2004). An obvious consequence of HD disruption is the delocalisation of α6β4 integrin, leading to a loss of its interaction with the intermediate filaments and therefore to a looseness of keratinocyte attachment to the extracellular matrix. However, the release of α6β4 integrin from its mechanical adhesive function correlates with its connection to the actin network at the lamellipodium, its association with tyrosine kinase receptors and with an increased signalling activity as a consequence (Rabinovitz et al., 1999; Hamill et al., 2009). All these events contribute to promote cell migration (Mercurio et al., 2001). Motogenic factors such as EGF and macrophage stimulating protein (MSP) (Margadant et al., 2008; Santoro et al., 2003) have been identified to induce HD dismantling. Several recent studies have established that phosphorylation of serine residues located in the connecting segment of β4 cytodomain promotes the destabilisation of β4-plectin interaction and the release of β4 integrin from HD (Germain et al., 2009; Rabinovitz et al., 2004; Wilhelmsen et al., 2007). These phosphorylations could be regulated by multiple signalling pathways involving protein kinases such as protein kinase C (PKC) (Germain et al., 2009; Rabinovitz et al., 2004; Wilhelmsen et al., 2007), protein kinase A (PKA) (Wilhelmsen et al., 2007). More recently, Frijns and colleagues have shown that in response to EGF, ERK1/2 and its effector p90 ribosomal S6 kinase (p90RSK) phosphorylate β4 integrin on serine residues that are critical for the interaction between β4 integrin and plectin (Frijns et al., 2010). Importantly, prevention of β4 integrin phosphorylation, by directed mutagenesis of the serine residues that promotes HD-like structure formation and stabilisation is sufficient to slow cell migration (Frijns et al., 2010; Kashyap et al.,

2011). Aside from these data, no information is yet available concerning extracellular cues or intracellular signalling pathways that may promote or stabilise β4 integrin localisation into HD.

During skin wound healing, keratinocyte migration is orchestrated by EGF and numerous other growth factors as well as cytokines and newly produced extracellular matrix proteins (Kirfel and Herzog, 2004). Extracellular nucleotides are also released at micromolar concentrations in the wound bed (Lazarowski et al., 2003; Holzer and Granstein, 2004; Burrell et al., 2003; Yin et al., 2007). At these concentrations, extracellular nucleotides activate two classes of purinergic receptors: the G-protein-coupled receptors (P2Y) and the ATP-gated ion channels (P2X) (Burnstock, 2007). The UTP receptor P2Y2R triggers cell migration in different cell types (Chaulet et al., 2001; Bagchi et al., 2005; Kaczmarek et al., 2005; Yu et al., 2008; Pillois et al., 2002; Wang et al., 2005; Chen et al., 2006). In particular, extracellular nucleotides stimulate cell migration in wounded corneal epithelium via P2Y2R activation (Boucher et al., 2010; Boucher et al., 2011; Yin et al., 2007). However, in contrast with these data, we previously showed that P2Y2R inhibits serum-induced keratinocyte migration through activation of the heterotrimeric Gαq protein (Taboubi et al., 2007). More recently, we reported that P2Y2R inhibits the insulin-like growth-factor-induced keratinocyte migration by blocking PI3K activation (Taboubi et al., 2010). However, in addition to PI3K pathway, our work provides some clues suggesting that extracellular UTP may also be able to dampen keratinocyte migration by a negative crosstalk with other signalling pathways (Taboubi et al., 2007).

In the present work, we used HaCaT keratinocyte cell line that forms HD-like structures enriched in α6β4 integrin and others HD components similar to those found in the epidermis basal epithelial layer (Geuijen and Sonnenberg, 2002; Tsuruta et al., 2003; Hamill et al., 2009). We report that the activations of ERK1/2 signalling pathway and its effector p90RSK are required for HD disruption by EGF. We further demonstrate that stimulation of P2Y2R and Gαq by UTP initiates a signal that inhibits EGF-induced ERK1/2 activation downstream of the Raf kinase. Remarkably, P2Y2R/Gαq activation counteracts EGF signal transduction to stabilise α6β4 integrin, plectin, BPAG1, BPAG2 and CD151 into HD plaques. These data provide the first evidence of an interconnected signal between MAPK/p90RSK and P2Y2R leading to the regulation of HD dynamics and keratinocyte migration.

Results

P2Y2R inhibits EGF-induced ERK1/2-dependent keratinocyte migration

Epidermal growth factor (EGF) is a potent inductor of keratinocyte migration through the activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase 1/2 (ERK1/2) pathway (Haase et al., 2003). In order to examine the effects of extracellular UTP on EGF-induced HaCaT cell migration, we performed random two-dimensional cell motility assays using video-microscopy (Taboubi et al., 2010). In agreement with previous work (Haase et al., 2003), EGF treatment enhanced HaCaT cell migration (Fig. 1A). Quantitative analysis of the individual cell trajectories for 2 h indicated that EGF increased the mean cell speed by two fold (0.52±0.02 μm/min vs 0.96±0.03 μm/min for control and EGF, respectively) and did not change cell directionality (data not shown). As expected, inhibition of MEK1/2 with the pharmacological inhibitors PD98059 and U0126 significantly reduced basal and EGF-induced cell migration

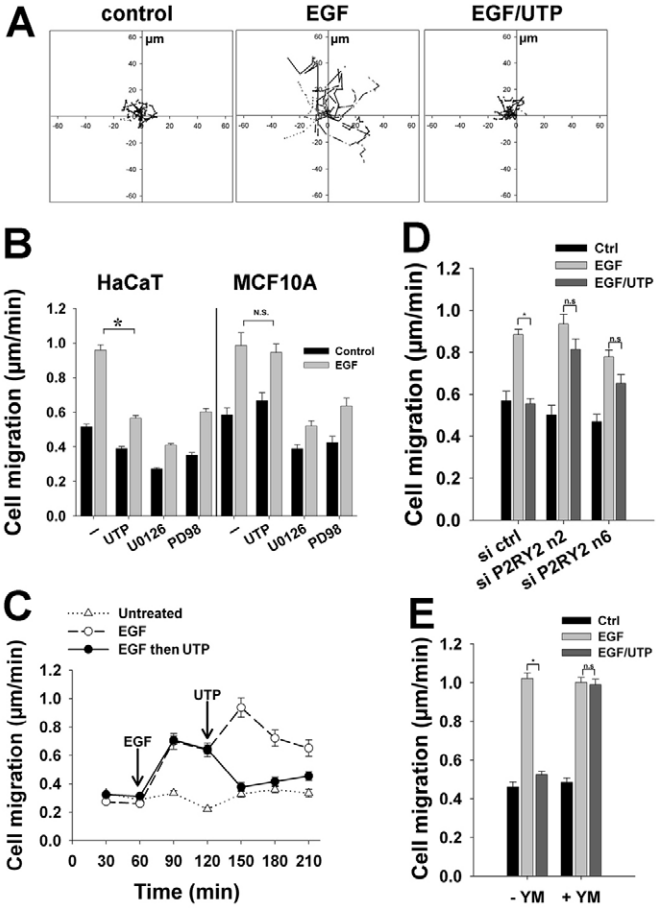

Fig. 1. UTP inhibits EGF-induced cell migration in HaCaT cells through P2Y2R and Gαq. (**A**) HaCaT keratinocytes were either left untreated (control) or treated with EGF (10 nM) alone (EGF) or in the presence of 100 μM UTP (EGF/UTP). The migration paths of 10 cells that were followed for 120 min are represented. (**B**) HaCaT or MCF10A cells were pretreated for 45 min, before migration assay, without (−) or with U0126 (U0126, 10 μM) or PD98659 (PD98, 20 μM), two specific pharmacological inhibitors of MEK1/2. Cells were either left unstimulated (Control; black bars) or treated with 10 nM EGF (EGF, grey bars) and migration assays were performed for 120 min. Cell velocity was quantified (μm/min) as described in Materials and Methods. Values are means ± s.e.m.; $n=3$ (>100 cells per experiment); $^*P<0.001$. (**C**) HaCaT cells were either left untreated (open triangles) or treated with EGF alone (open circles) or treated with EGF and then UTP (black circles). Individual keratinocyte migration was monitored for 210 min. When indicated, 100 μM UTP was added simultaneously with EGF (UTP). Data are expressed as the mean velocity of individual cells calculated over a period of 30 min. These data are representative of three individual experiments. Values are means ± s.e.m.; $n>150$ cells per experiment. (**D**) HaCaT cells were transfected with control siRNA (si ctrl) or siRNA targeting P2Y2R (si P2Y2Rn2, si P2Y2Rn6) as described in Materials and Methods. Control for siRNA efficiency is presented in supplementary material Fig. S1. Forty-eight hours after transfection, cell migration analysis was performed on untreated cells (Ctrl) or cells treated with EGF (10 nM) in the absence (EGF) or the presence of 100 μM UTP (EGF/UTP). Data are expressed as the mean cell velocity obtained from several independent experiments (>150 cells per experiment). Values are means ± s.e.m.; $n=3$; $^*P<0.001$. (**E**) HaCaT cells were preincubated for 45 min without (−YM) or with (+YM) 1 μM YM-254890, a Gαq pharmacological inhibitor. Cells were then either left untreated (Ctrl) or treated with 10 nM EGF either alone (EGF) or in the presence of 100 μM UTP (EGF/UTP). Data are expressed as the mean cell velocity obtained from 3 independent experiments (>150 cells per experiment). Values are means ± s.e.m.; $n=3$; $^*P<0.001$.

(Fig. 1B). Remarkably, UTP (100 μM) reduced EGF-induced HaCaT cell velocity by 1.6 fold (0.96±0.03 μm/min and 0.57±0.02 μm/min for EGF- and EGF/UTP-treated cells, respectively, $P<0.01$; Fig. 1B). Inhibition of HaCaT cell migration by UTP was sustained and persistent even 6 h after treatment (data not shown). In other series of experiments, UTP was able to stop an ongoing EGF-induced HaCaT cell migration even when added 1 h after EGF (Fig. 1C).

UTP has been described as a motogenic factor in other epithelial cell type (Klepeis et al., 2004; Yin et al., 2007; Boucher et al., 2010). Thus, we tried to determine whether the antimotogenic UTP effects were restricted to keratinocytes. Therefore, we used the same migration assays to test the effects of EGF and UTP on a human mammary epithelial cell line (MCF10A). These cells express P2Y2R as a functional UTP receptor as determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and measurement of UTP-induced intracellular calcium flux (data not shown). As shown for HaCaT cells, and in agreement with previous published data (Joslin et al., 2007), EGF stimulated MCF10A cell migration in an ERK1/2-dependent manner (0.6±0.04 μm/min and 0.99±0.07 μm/min for control and EGF-treated cells, respectively; Fig. 1B). However, by contrast with HaCaT cells, UTP failed to inhibit the EGF-induced MCF10A migration (0.99±0.007 μm/min and 0.95±0.05 μm/min for EGF- and EGF/UTP-treated cells, respectively; Fig. 1B). These data indicate that the anti-motogenic signals triggered by UTP are not a common feature shared by all epithelial cells and may be restrained to specific cell types such as keratinocytes.

In keratinocytes, the ATP/UTP-sensitive Gαq-coupled P2Y2R is the main functional receptor for UTP (Inoue et al., 2007; Taboubi et al., 2007; Taboubi et al., 2010; Yoshida et al., 2006; Koizumi et al., 2004; Lee et al., 2001). In order to confirm that UTP effects on HaCaT cell migration are mediated by P2Y2R, motility assays were repeated with cells transfected with siRNA targeting P2Y2R. The efficiency of the two siRNA sequences used in this study was validated by qRT-PCR (supplementary material Fig. S1). As shown in Fig. 1D, silencing of P2Y2R strongly desensitises cells to UTP effects. Using a Gαq pharmacological inhibitor (YM-254890) (Takasaki et al., 2004), we confirmed that Gαq activation was also required for an efficient inhibition of keratinocyte migration by UTP (Fig. 1E). Therefore, our data establish that UTP activates P2Y2R and Gαq to inhibit EGF-induced HaCaT cell migration.

P2Y2R signalling inhibits EGF-induced ERK1/2 activation

To gain further insight into the mechanism involved in UTP-induced inhibition of keratinocyte migration, we examined extracellular UTP effects on ERK1/2 activation by EGF. We monitored ERK1/2 phosphorylation kinetics upon EGF stimulation, in the presence or the absence of UTP, in HaCaT and MCF10A cells. EGF stimulation promoted ERK1/2 phosphorylation in both cell lines (Fig. 2A,B). It should be noted that ERK1/2 stimulation by EGF was stronger in HaCaT cells than in MCF10A cells. When UTP was added simultaneously with EGF, UTP strikingly prevented ERK1/2 phosphorylation induced by EGF in HaCaT cells (Fig. 2A) while it was unable to inhibit ERK1/2 phosphorylation in MCF10A cells (Fig. 2B). However, this inhibition was transient and decreased 30 min after treatment. In agreement with previous reports on keratinocytes (Kobayashi et al., 2006; Giltaire et al., 2011), in absence of any growth factor UTP induced ERK1/2 phosphorylation 30 min after its addition to the culture medium. Thus, UTP was able to elicit dual opposite signal towards ERK1/2: a conventional stimulatory signal and an unusual inhibitory signal revealed when HaCaT are stimulated by EGF and UTP.

As shown in Fig. 2C, a siRNA-mediated silencing approach revealed the involvement of P2Y2R in the UTP-mediated ERK1/2 inhibition in HaCaT cells. Gαq involvement in ERK1/2 inhibition by UTP was then addressed by using either siRNA targeting Gαq (Fig. 2D) or the pharmacological inhibitor YM-254890 (Fig. 2E). These data show that UTP inhibited ERK1/2 phosphorylation through P2Y2R and Gαq activation.

It has been reported that in human endothelial cells ERK1/2 activation by extracellular UTP is anchorage dependent (Short et al., 2000). By contrast, P2Y2R signalling pathway leading to ERK1/2 inhibition was maintained in suspended cells co-stimulated by UTP and EGF (supplementary material Fig. S2). Thus, our data highlight a new anchorage independent signal transduction pathway triggered by the UTP/P2Y2R/Gαq axis that impedes ERK1/2 activation by EGF in HaCat cells.

UTP inhibits the ERK1/2 pathway downstream of Raf

The first step in the signal transduction initiated by EGFR is its autophosphorylation on tyrosine residues (Wells, 1999). Therefore, we examined the phosphorylation level of four different EGFR tyrosine residues upon EGF, UTP or EGF/UTP treatments. As shown in Fig. 3A, UTP did not induce EGFR phosphorylation on any tyrosine residue tested in these experiments in HaCaT cells. Thus, in sharp contrast with data reported in other cell types (Ratchford et al., 2010; Liu et al., 2004; Morris et al., 2004; Soltoff, 1998; Boucher et al., 2011), P2Y2R was not able to transactivate EGFR in keratinocytes. When added simultaneously with EGF, UTP had no significant impact on the phosphorylation level of Tyr845, Tyr992, Tyr1045 and Tyr1068 upon EGF stimulation (Fig. 3A). Importantly, it has been reported that Tyr992 and Tyr1068 are two essential tyrosine residues for ERK1/2 activation via the engagement of the phospholipase type Cγ and the growth factor receptor-bound protein 2 (Grb2), respectively (reviewed in Olayioye et al., 2000). Thus, our results show that UTP is unable to affect the phosphorylation level of EGF receptor tyrosine residues involved in the activation of ERK1/2 pathway in HaCaT cells.

Downstream of EGFR, the serine/threonine protein kinase Raf is the first kinase of the MAPK signalling cascade. Among the three members of Raf kinase family, Raf-1 (also called c-Raf) has been shown to be required for in vitro keratinocyte migration and for efficient wound healing in vivo (Ehrenreiter et al., 2005). Raf-1 phosphorylation on the activating Ser338 (Diaz et al., 1997) was detectable in HaCaT cells within minutes following EGF stimulation, but it was not altered by UTP signalling (Fig. 3B). In contrast, at these time points, MEK and ERK1/2 phosphorylations were strongly inhibited by UTP (Fig. 3B). UTP also dampen the phosphorylation level of the transcription factor Elk-1, a direct downstream target of ERK1/2 (Fig. 3B). Taken together, these results suggest that UTP may downregulate MAPK signalling cascade downstream of Raf-1. To substantiate this, we further used ΔA-Raf:ER HaCaT cells that were engineered to express an inducible A-Raf–estrogen receptor fusion protein (Rössler and Thiel, 2004; McMahon, 2001). In these experiments, ERK1/2 pathway was activated using 4′-hydroxytamoxifen (4′OHT) or EGF treatments, 30 min before addition of UTP. As shown in Fig. 3C (upper panel), the addition of 4′OHT induced ERK1/2 phosphorylation, indicating a potent activation of the ΔA-Raf:ER–MEK–ERK1/2 pathway. UTP decreased ERK1/2 phosphorylation

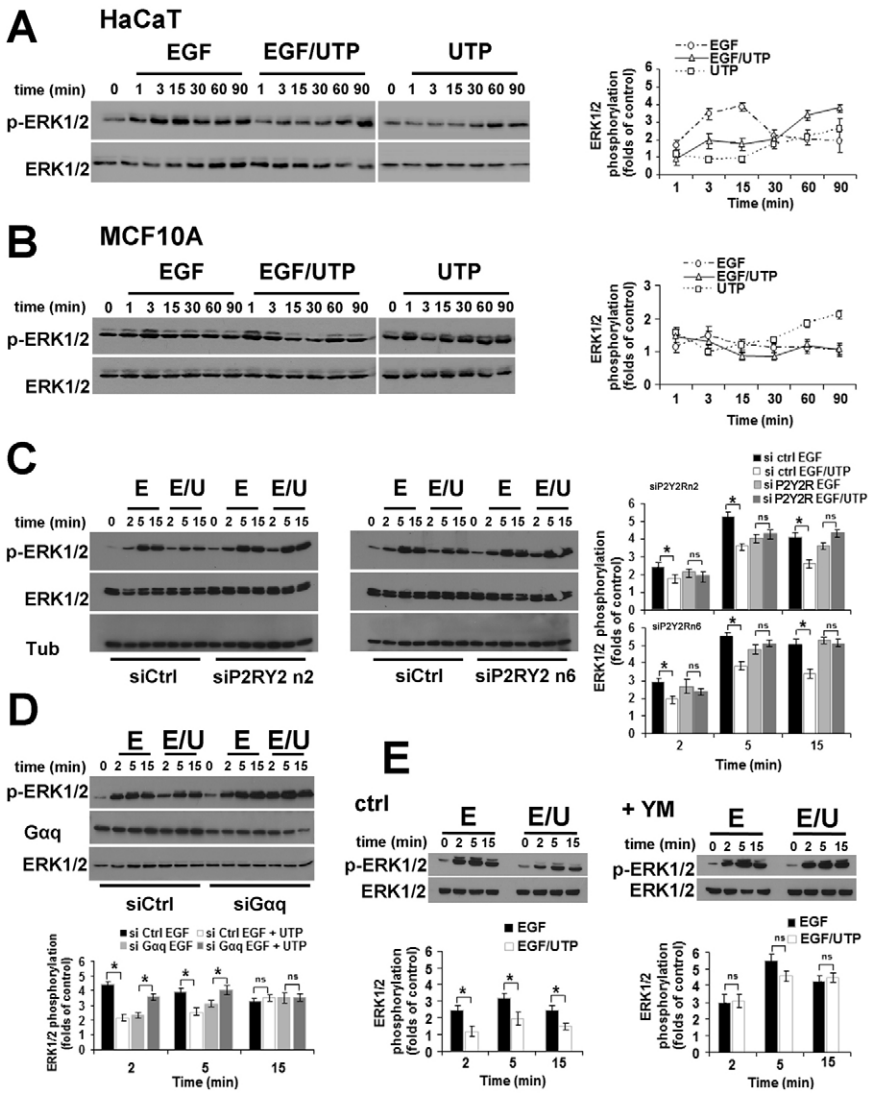

Fig. 2. UTP inhibits the EGF-induced ERK1/2 signalling pathway through P2Y2R in HaCaT cells. HaCaT cells (**A**) or MCF10A cells (**B**) were stimulated for the indicated times with either 10 nM EGF (EGF), 100 μM UTP (UTP) or both (EGF/UTP). Cells were then lysed and protein contents were analysed by immunoblotting using anti-phospho-ERK1/2 (p-ERK1/2) and anti-ERK1/2 (ERK1/2) antibodies. Graph show the fold increase of ERK phosphorylation (p-ERK/ERK) normalised to control (untreated cells); $n=3$; $^*P<0.05$. (**C**) HaCaT cells were transfected with control siRNA (siCtrl) or siRNA against P2Y2R (siP2Y2Rn2 or siP2Y2Rn6). Cells were then stimulated with either 10 nM EGF alone (E) or in the presence of 100 μM UTP (E/U) for the indicated times. Cells were then lysed and protein contents were analysed by immunoblotting using anti-phospho-ERK1/2 (p-ERK1/2), anti-ERK1/2 (ERK1/2) and anti-tubulin (Tub) antibodies. Controls for siRNA efficiency are presented in supplementary material Fig. S1. Histograms show the fold increased of ERK phosphorylation (p-ERK/ERK) normalised to control, untreated cells ($n=3$; $^*P<0.05$). (**D**) HaCaT cells were transfected with control siRNA (siCtrl) or with siRNA against Gαq (siGαq) as described in Materials and Methods. Cells were then stimulated with either EGF alone (E, 10 nM) or in the presence of 100 μM UTP (E/U) for the indicated times. After cell lysis, protein contents were analysed by immunoblotting using anti-phospho-ERK1/2 (p-ERK1/2), anti-Gαq and anti-ERK1/2 (ERK1/2) antibodies. Histograms show the fold increase of ERK phosphorylation (p-ERK/ERK) normalised to control, untreated cells ($n=3$; $^*P<0.05$). (**E**) HaCaT cells were pretreated for 45 min without (ctrl) or with 1 μM YM-254890 (+YM), a pharmacological inhibitor of Gαq protein. Cells were then stimulated with either EGF alone (E, 10 nM) or in the presence of 100 μM UTP (E/U) for the indicated times. Histograms show the fold increase of ERK phosphorylation (p-ERK/ERK) normalised to control, untreated cells ($n=4$; $^*P<0.05$).

induced by ΔA-Raf:ER activation within a few minutes. As a control, immunoblots presented in Fig. 3C (lower panel) show that UTP also reduced EGF-induced ERK1/2 phosphorylation in ΔA-Raf:ER HaCaT cells. Taken together, these data indicate that UTP efficiently inhibits EGF-induced ERK1/2 pathway activation downstream of Raf kinase.

Activation of the ERK1/2 pathway is necessary for EGF-induced HD disruption

As mentioned above, initiation of epithelial cell migration by EGF requires a profound remodelling of stable adhesion sites, notably HD (Margadant et al., 2008). Upon EGFR activation, the phosphorylation of serine residues on β4 integrin cytoplasmic domain is an essential step of integrin/plectin dissociation and HD disassembly (Germain et al., 2009; Rabinovitz et al., 2004; Wilhelmsen et al., 2007; Frijns et al., 2010; Frijns et al., 2012). Importantly, directed mutagenesis of these serine residues, which prevents β4 integrin phosphorylation, slows cell migration of keratinocytes and squamous cell carcinoma (Frijns et al., 2010; Kashyap et al., 2011). As ERK1/2 signalling pathway was shown to participate to the phosphorylation of β4 integrin downstream of EGFR (Frijns et al., 2010), we sought to verify whether ERK1/2 is involved in the regulation of the EGF-dependent mobilisation of β4 integrin from HD in HaCaT cells.

For this purpose, serum-starved HaCaT cells were pretreated with various inhibitors of the Raf/MEK/ERK1/2 pathway before EGF stimulation. As previously reported by others (Ozawa et al., 2010), untreated HaCaT cells displayed a classical ‘leopard-skin’ pattern enriched in β4 integrin, characteristic of HD-like structures (Fig. 4A). Similar patterns were observed with other HD components namely plectin, CD151, BPAG1, BPAG2 and laminin-332 (supplementary material Fig. S3), indicating that these adhesive structures resemble hemidesmosomes found in vivo. As expected, upon EGF stimulation β4 integrin was diffusely distributed (Fig. 4A). Similar observations were made with plectin, CD151, BPAG1 and BPAG2 confirming that EGF provoked HD plaques disassembly (supplementary material Fig. S3). EGF had no noticeable impact on laminin-332 pattern (supplementary material Fig. S4). Moreover, we controlled by fluorescence activated cell sorting experiments that EGF did not decrease β4 integrin expression (supplementary material Fig. S5). Quantitative analyses were performed to assess the amount of cells expressing β4 integrin

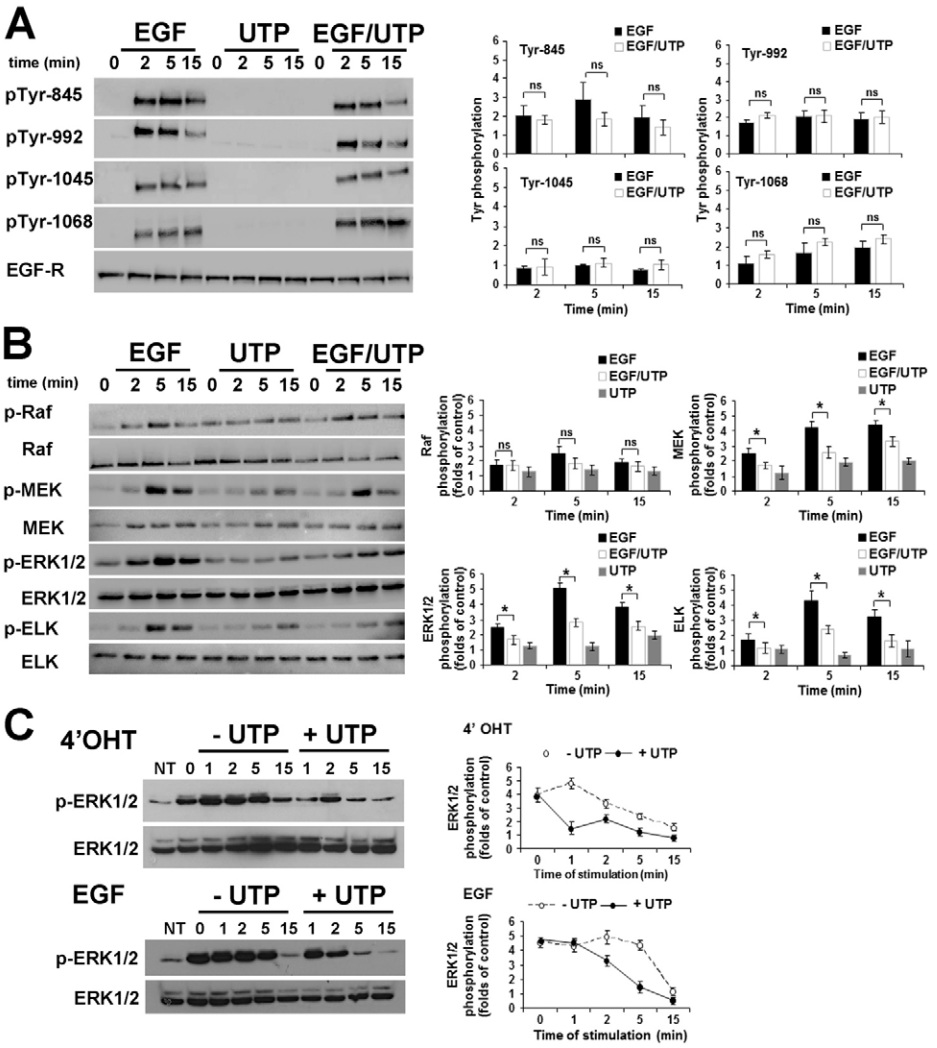

Fig. 3. UTP inhibits the ERK1/2 signalling pathway downstream of Raf. (**A**) HaCaT cells were stimulated for the indicated times with 100 μM UTP, 10 nM EGF alone or in combination (EGF/UTP). Cell lysates were analysed by western blotting using antibodies against various phosphotyrosine sites of EGF receptor: anti-phospho-Tyr845 (pTyr-845), anti-phospho-Tyr992 (pTyr-992), anti-phospho-Tyr1045 (pTyr-1045), anti-phospho-Tyr1068 (pTyr-1068) or anti-EGF receptor (EGF-R) antibodies as indicated. Phospho-Tyr845, phospho-Tyr992, phospho-Tyr1045 and phospho-Tyr1068 were detected as described in Materials and Methods and quantified by densitometry. Histograms show the fold increase of phosphorylation normalised to control, untreated cells (n=5; ns, non-significant, P>0.05). (**B**) HaCaT cells were stimulated for the indicated times with 100 μM UTP, 10 nM EGF alone or with both (EGF/UTP). After cell lysis, immunoblottings were performed using anti-phospho Raf-1 (p-Raf), anti-phospho-MEK1/2 (p-MEK), anti-phospho-ERK1/2 (p-ERK1/2), anti-phospho-Elk1 (p-ELK), anti-Raf-1 (Raf), anti-MEK1/2 (MEK), anti-ERK1/2 (ERK1/2) and anti-Elk1 (ELK) antibodies as indicated. Histograms show the fold increase of protein phosphorylation normalised to control, untreated cells (n=4; *P<0.05). (**C**) For the duration of the experiment, HaCaT ΔA-RAF:ER cells were maintained in suspension and pretreated for 30 min with 100 nM 4′-hydroxitamoxifen (4′OHT) or with 10 nM EGF (EGF). 100 μM UTP was then added to cell suspension for 1, 2, 5 or 15 min. Cell lysates were analysed by western blotting using anti-phospho-ERK1/2 (p-ERK1/2) and anti-ERK1/2 antibodies. NT refers to cells kept in depleted medium during the experiments. Graphs show the fold increase of ERK phosphorylation normalized to control, untreated cells (n=3; *P<0.05).

in HD-like adhesion sites. Kinetic analysis of the number of cells with dense β4 integrin leopard-skin pattern revealed that optimal HD disruption was obtained 45 min after EGF addition (supplementary material Fig. S6). At this time point, EGF induced a 3-fold decrease in the number of cells harbouring β4-enriched HD plaques (79.9±3.4% and 28.5±2.7% for control and EGF-treated cells, respectively; Fig. 4B). Pharmacological inhibition of either MEK1/2 or Raf-1 reversed EGF-induced β4 mobilisation from HD (57.9±12.7%, 59.4±2.4% and 70.2±3.5% of HD-positive cells in presence of PD98059, U0126 and Raf inhibitor, respectively), without significant impact on β4 integrin distribution in serum-starved HaCaT cells (Fig. 4B). To further extend this observation, we repeated the same experiments with two different epithelial cell lines: MCF10A cells, which express β4 integrin in typical HD-like structures (Stahl et al., 1997), and A431 cells, which derived from an epidermoid carcinoma and present HD-like structure sensitive to EGF treatment (Rabinovitz et al., 1999). As shown in Fig. 4C,D, the addition of MEK inhibitors prevented HD remodelling by EGF in both cell lines. Thus, in agreement with data reported by Frijns and co-workers (Frijns et al., 2010), our results confirm that ERK1/2 is fully involved in the regulation of β4 integrin dynamics and HD dismantling by EGF in epithelial cells.

P2Y2R signalling stabilises HD and promotes their formation

The results reported above suggest that an inhibition of the EGF-induced MAPK signalling pathway by UTP may reflect an opposite regulation of HD stabilisation by EGF and UTP. To evaluate the putative role of P2Y2R signalling in HD dynamics, we first examined whether UTP can impede HD disruption by EGF. Confocal images revealed that EGF failed to promote mobilisation of β4 integrin from HD when it was added at the same time as UTP (Fig. 5A). The number of EGF/UTP-treated cells exhibiting β4 integrin-positive HD plaques was maintained at the level of control serum-starved cells (63.2±3.5%, 24.9±3.5% and 58.6±3.0%, for control, EGF- and EGF/UTP-treated cells, respectively). Note that fluorescence activated cell sorting experiments revealed that UTP had no impact on β4 integrin expression (supplementary material Fig. S5). Importantly, co-labelling of α6 integrin subunit with other HD components showed that, UTP impeded the redistribution of CD151, plectin, BPAG1 and BPAG2 induced by EGF (supplementary material Fig. S3), but had no noticeable effect on laminin-332 pattern (supplementary material Fig. S4). As revealed by time course experiments, we observed that UTP stabilised β4 in HD-like plaques for up to 3 h (supplementary

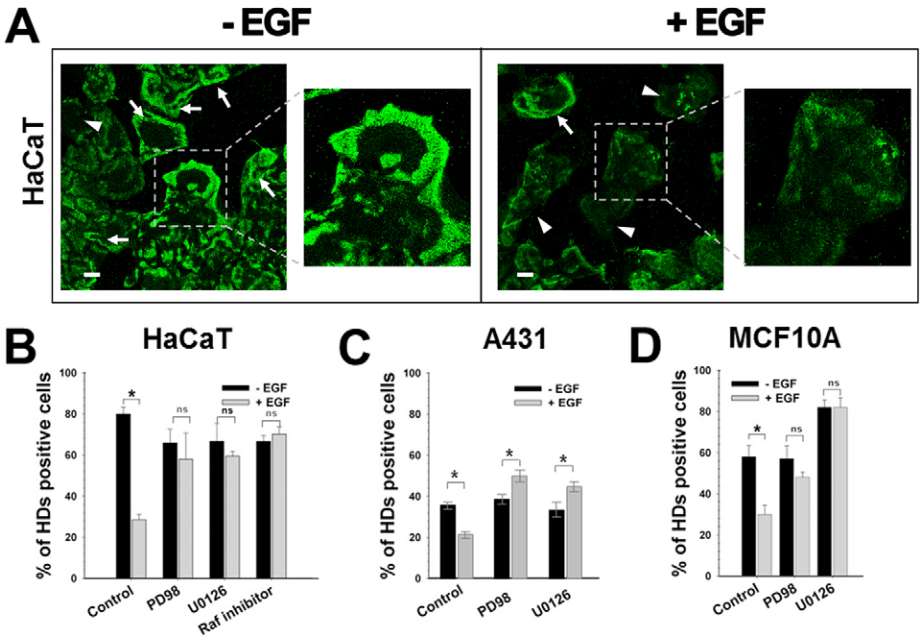

Fig. 4. EGF-induced HD disruption involves the MAPK signalling pathway in HaCaT cells. (**A**) HaCaT cells seeded on collagen-I were stimulated for 45 min with (+EGF; 10 nM) or left untreated (−EGF). HDs were visualised by confocal immunofluorescence microscopy using an anti-β4 integrin antibody (scale bar: 20 μm). Arrows indicate cells considered as 'HD-positive cells' with the characteristic 'leopard spots' β4 integrin staining. Arrowheads indicate cells considered as 'HD-negative cells' in which β4 integrin staining is more diffuse. (**B–D**) Serum-starved HaCaT (B), A431 (C) and MCF10A (D) cells were either left untreated (Control) or preincubated for 45 min with PD98059 (PD98, 20 μM) or U0126 (10 μM), two pharmacological MEK1/2 inhibitors, or with a Raf-1 inhibitor (Raf inhibitor, 10 μM). Cells were then stimulated for 45 min with EGF (+EGF, 10 nM; grey bars) or left unstimulated (−EGF; black bars). The quantification of HD-positive cells was performed as described in Materials and Methods. Values are means ± s.e.m. of four independent experiments (>100 cells per experimental conditions); *$P<0.001$.

material Fig. S6). This highlights that extracellular UTP regulates HD-like structure dynamics in HaCat cells, and prevails over EGF to stabilise these adhesion sites.

To assess whether P2Y2R transduces UTP signal leading to HD stabilisation, we examined β4 integrin distribution in HaCaT cells in which P2Y2R expression was repressed by synthetic siRNAs. As shown in Fig. 5B, the proportion of HaCaT cells transfected with control siRNA (siCtrl) bearing HD plaques reached 68.9±2.2% compared to only 30±0.2% in the presence of EGF. In agreement with data presented in Fig. 5A, UTP completely inhibited EGF-induced mobilisation of β4 integrin from HD (72±4.1% of HD-positive cells). By contrast, treatment with two different siRNA targeting P2Y2R abolished HD stabilisation by UTP (42.7±3% and 38.2±3.9% of cells with HD plaques for siP2Y2Rn2 and siP2Y2Rn6, respectively; Fig. 5B). The role of Gαq protein in the signal pathway transduced by P2Y2R leading to HD stabilisation was further investigated using the Gαq pharmacological inhibitor YM-254890 (Fig. 5C). Clearly, UTP was no longer able to stabilise β4 integrin in HD plaques in EGF-treated cells when Gαq was inhibited (59.9±8.5% and 33.9±6.3% of HD-positive cells, in the absence or the presence of YM-254890, respectively). Finally, we seek to determine whether this remarkable property of UTP was also observed in other epithelial cells. It has been previously reported that A431 cells express a functional P2Y2 receptor (Greig et al., 2003). As observed in HaCaT cells, UTP was also able to prevent β4 redistribution by EGF in these cells (Fig. 5D). By contrast, UTP had no impact on the HD dismantling in MCF10A cells (Fig. 5E), thus suggesting that the stabilisation of HD-like structures by P2Y2R signalling may be restricted to keratinocyte-derived cells.

Having established that UTP can stabilise HD-like plaques in HaCat cells via P2Y2R and Gαq, we next examined whether UTP could promote formation of these adhesives complexes once they have been disrupted by EGF. To answer this question, cells were initially treated with EGF for 60 min to disrupt HD plaques (reduction of the number of HD-positive cells from 80% to about 40%). Then EGF was withdrawn from the medium and HD formation was monitored. As shown in Fig. 6A,B, the amount of cells with dense β4 integrin labelling in HD-like structures remained stable for 30 min after EGF washout. However, a slight increase was observed 2 h after EGF withdrawal. This illustrated that initial signals elicited by EGF were sufficient to durably inhibit HD-like structure formation. Importantly, when UTP was added after EGF washout, the rate of β4 integrin relocalisation within HD-like structures was greatly enhanced. Already observed 15 min after UTP addition (around 58% of HD-positive cells), HD formation was almost completed 30 min after UTP treatment. These neo-formed HD plaques were stable and present in nearly 80% of the cells 2 h after UTP addition (Fig. 6A,B).

Together, our data show that UTP and P2Y2R play important regulatory functions in HD dynamics: they can both prevent HD disassembly by EGF and stimulate HD formation.

UTP blocks p90RSK function to promote HD stability

p90RSK (ribosomal S6 kinase) has been recently shown to be an essential kinase mediating ERK1/2 motogenic signalling function (Doehn et al., 2009; Smolen et al., 2010). Moreover, data from

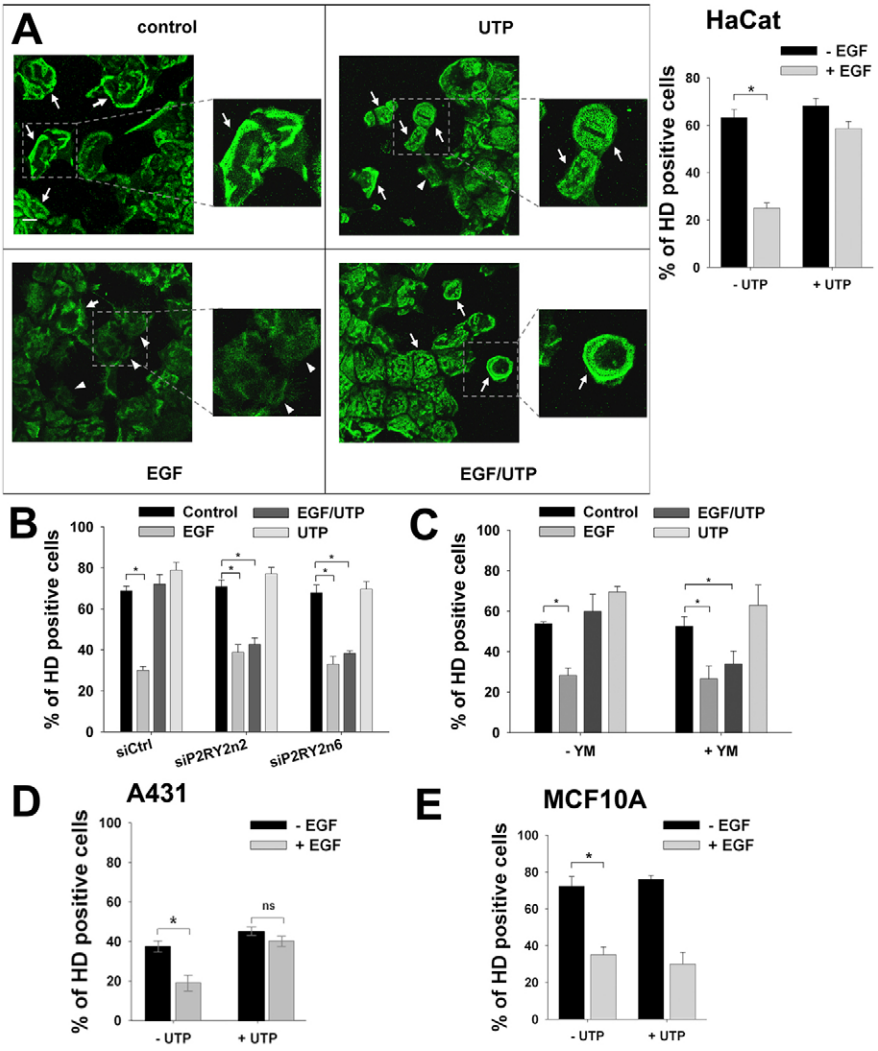

Fig. 5. UTP inhibits EGF-induced β4 integrin mobilisation from HD in HaCaT cells through P2Y2R and Gαq protein. (**A**) HaCaT cells seeded on collagen-I were stimulated for 45 min with EGF (10 nM), either alone or with UTP (100 μM). HDs were visualised by confocal immunofluorescence using an anti-β4 integrin antibody. The quantification of HD-positive cells (right panel) was performed as described in Materials and Methods. Arrows indicate cells considered as HD-positive cells with the characteristic 'leopard spots' β4 integrin staining. Arrowheads indicate cells considered as 'HD-negative cells' in which β4 integrin staining is more diffuse. Values are means ± s.e.m. of five independent experiments (>100 cells per experimental conditions); **P*<0.001. (**B**) HaCaT cells were transfected with control siRNA (siCtrl) or with siRNA against P2Y2R (siP2Y2Rn2 or siP2Y2Rn6) as described previously. Twenty-four hours after transfection, cells were seeded on collagen-I. After serum starvation, cells were either left unstimulated (Control) or treated with 10 nM EGF, 100 μM UTP or with both EGF and UTP (EGF/UTP) for 45 min. HD were visualised by confocal immunofluorescence using an anti-β4 integrin antibody. Quantification of HD-positive cells was performed as previously described. The values are means ± s.e.m. of three independent experiments (>100 cells per experimental conditions); **P*<0.001. (**C**) HaCaT cells were left untreated (−YM) or preincubated with YM-254890 (1 μM; + YM) for 45 min. Cells were then either left untreated (Control) or treated with 10 nM EGF or 100 μM UTP or with both EGF and UTP for 45 min. HD were visualised by confocal immunofluorescence microscopy using an anti-β4 integrin antibody. Quantification of HD-positive cells was performed as previously described. Values are means ± s.e.m.; $n=3$ (>100 cells per experimental conditions); **P*<0.001. (**D**,**E**) A431 (D) and MCF10 cells (E) were stimulated for 45 min with 10 nM of EGF (+EGF) either alone or in the absence (−UTP) or in the presence of 100 μM UTP (+UTP) as indicated. The quantification of HD positive cells was performed as described in Materials and Methods. The values are means ± s.e.m. of four independent experiments (>100 cells per experimental conditions); **P*<0.001. Scale bar: 20 μm.

Sonnenberg's group showed that the phosphorylation state of serine residues, located in β4 integrin cytoplasmic domain and substrates of p90RSK, is critical for β4 integrin localisation in HD (Frijns et al., 2010). Thus, we hypothesise that purinergic receptors may inhibit p90RSK activation and function downstream of MEK and ERK1/2 in keratinocytes.

Using a selective p90RSK1/2 inhibitor, BID-1870 (Sapkota et al., 2007), we first aimed to establish whether p90RSK was involved in the control of HaCaT cell migration. As shown in Fig. 7A, stimulation of HaCaT cell migration by EGF was strongly reduced after BID-1870 treatment, thus confirming that p90RSK played a central function in the motogenic signals elicited by the EGF/ERK1/2 axis. Membrane targeting is an important step of p90RSK activation process (Richards et al., 2001). Here, we show that expression of a membrane targeted myr-p90RSK mutant is not sufficient to stimulate HaCaT cell migration either in the absence or in the presence of exogenous EGF (Fig. 7C), suggesting that p90RSK activation at the plasma

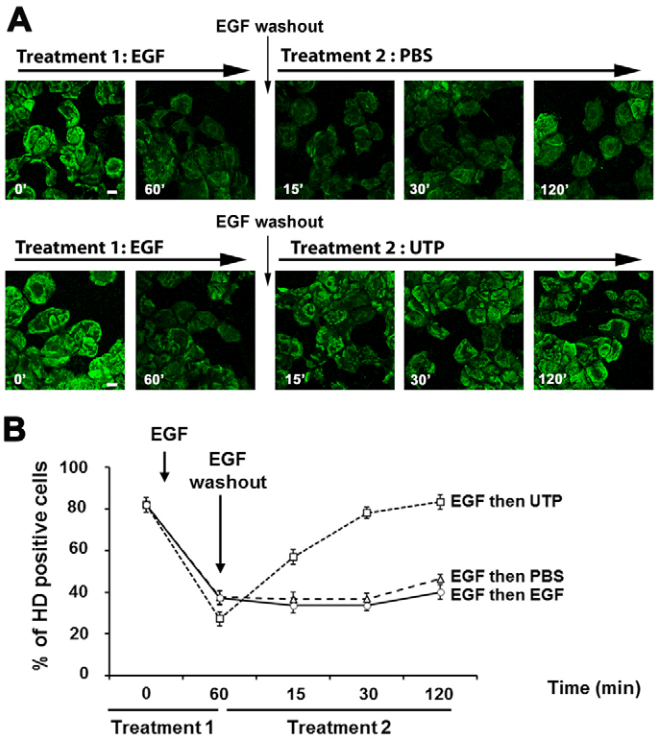

Fig. 6. UTP stabilises and promotes HD formation. (**A**) HaCaT cells plated on collagen-I were stimulated for 60 minutes with EGF (10 nM; EGF 60′, Treatment 1). The culture medium was changed (EGF washout) and cells were treated with PBS, UTP (100 µM; UTP) or EGF (10 nM; EGF) for various times as indicated (Treatment 2). HDs were visualised by confocal immunofluorescence analysis using an anti-β4 integrin antibody. Scale bars: 20 µm. Pictures show β4 integrin immunofluorescence of cells treated with EGF (60′) followed by PBS (upper panel) or UTP (lower panel). (**B**) The quantifications of HD-positive cells were performed as described in the legends of Figs 4 and 5. Values are means ± s.e.m.; $n=3$ (>100 cells per experimental conditions); $^{*}P<0.001$.

membrane is not sufficient to promote epithelial cell migration. However, it should be noted that a different p90RSK active mutant has been reported to stimulate epithelial cell scattering (Doehn et al., 2009). This discrepancy may reflect differences in the potency of the two mutants to induce the transcription of motile genes.

We then examined the impact of P2Y2R signalling on p90RSK function. Data presented in Fig. 7D revealed that extracellular UTP dampened EGF-induced p90RSK phosphorylation on serine 380, a critical activation site (Anjum and Blenis, 2008). Importantly, it is known that membrane targeting of p90RSK is sufficient to trigger its phosphorylation on serine 380 (Richards et al., 2001). We showed that by contrast with the wild-type endogenous protein, myr-p90RSK mutant remained insensitive to the serine 380 phosphorylation inhibition by UTP (Fig. 7E), indicating that UTP signalling targets EGF signal transduction pathway upstream of p90RSK.

Next, we confirmed that in agreement with a previous report (Frijns et al., 2010) p90RSK inhibition by BID-1870 prevented EGF-induced β4 integrin mobilisation from HD plaques (Fig. 7F). Moreover, expression of myr-p90RSK was sufficient to prevent integrin β4 localisation in HD-like structures (Fig. 7G). Indeed, the amount of cells harbouring β4 integrin-enriched HD-like structures was strongly reduced following expression of myr-p90RSK (14.7±6.7% of the cells expressing high level of myr-p90RSK compared to 83.9±0.9% of the control cells that expressed low levels of myr-p90RSK). Finally, we took advantage of the dominant effect of the myr-p90RSK over the UTP signal transduction to analyse the impact of extracellular UTP on β4 integrin distribution in HaCaT cells expressing high level of myr-p90RSK. As shown in Fig. 7G, UTP failed to restore β4 integrin localisation in HD in myr-p90RSK-positive cells. Quantitative analysis showed no difference in the number of myr-p90RSK cells harbouring β4-integrin-positive HD plaques in the absence or in the presence of UTP (14.7±6.7% and 20.7±1.6%, respectively; Fig. 7G). Since p90RSK has been shown to enhance β4 integrin expression during epithelial–mesenchymal transition (Doehn et al., 2009), we controlled that the expression of myr-p90RSK did not change β4 integrin cell surface expression (data not shown) neither its total expression (Fig. 7E). Moreover, pharmacological inhibition of p90RSK by BID-1870 did not affect β4 integrin expression as determined by fluorescent cell sorting analysis of intact cells (supplementary material Fig. S5).

Thus, our data indicates that p90RSK activation at the membrane is necessary and sufficient to remove β4 integrin from HD. This should be linked with its capacity to phosphorylate β4 integrin cytoplasmic domain, in agreement with data reported by Frijns and co-workers (Frijns et al., 2010). Moreover, we provide strong evidence showing that in HaCat cells, UTP and EGF convey opposite signals that converge to modulate ERK/p90RSK pathway activation to control HD dynamics and cell migration.

Discussion

During wound re-epithelialisation, coordinated modulation of HD assembly and disassembly is critical for the regulation of keratinocyte migration. So far, most of the investigator efforts shed light on the regulation of HD disassembly by growth factors present in the wound bed such as EGF (Margadant et al., 2008). By contrast, only a few data are available on molecular mechanisms promoting HD formation or stabilisation during wound healing (Kopecki et al., 2009; Wang et al., 2010). In the present study, using a keratinocyte derived cell line (HaCat), we identified an unsuspected new signalling pathway elicited by extracellular nucleotides that promotes the formation and stabilisation of HD and, as a consequence, inhibits cell migration. We report that activation of P2Y2R and Gαq by UTP interferes with the MAPK pathway activation by EGF. The inhibition of ERK1/2 and its effector p90RSK by purinergic signalling triggers the reinforcement of β4 integrin localisation in HD. We propose that HD stabilisation by UTP causes the arrest of the EGF-induced ERK1/2/p90RSK-dependent cell motility (Fig. 8).

Our results highlight that UTP elicits a complex biphasic ERK1/2 regulatory signalling pathway in HaCat cells. At early time points, UTP is able to inhibit ERK1/2 phosphorylation by EGF. Using pharmacological inhibition and siRNA-mediated gene silencing, we show that this effect requires activation of P2Y2R, the main UTP receptor in keratinocytes (Taboubi et al., 2007) and the activation of Gαq protein (Fig. 2C–E). However, the inhibition of ERK1/2, detectable within the first minute of UTP addition, is transient, lasting for less than 30 min. At later time points, UTP triggers ERK1/2 phosphorylation (Fig. 2A). This observation is in agreement with previous studies carried out on keratinocytes

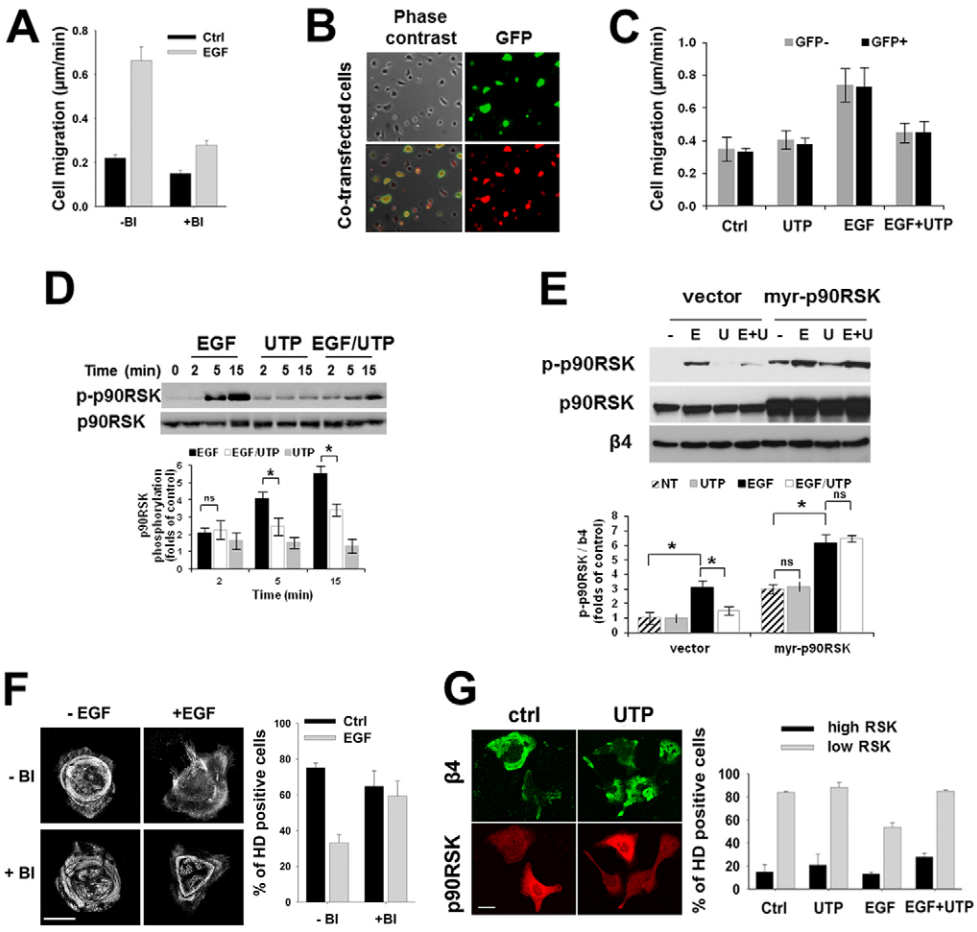

Fig. 7. p90RSK is required for EGF-induced cell migration and HD remodelling. (**A**) HaCaT cells were pretreated for 1 h, before a migration assay, without (−BI) or with 10 µM BID-1870 (+BI), a pharmacological inhibitor of p90RSK. Cells were either left unstimulated (Ctrl; black bars) or treated with 10 nM EGF (grey bars) and migration assay was performed for 120 min. Cell velocity was quantified (µm/min) as described in Materials and Methods. Values are means ± s.e.m., $n=3$ (>100 cells per experiment); *$P<0.001$. (**B**,**C**) HaCaT cells were co-transfected with a myr-p90RSK plasmid (myr-p90RSK) and with a plasmid coding for GFP protein (GFP). Immunostaining of co-transfected cells using p90RSK antibody is presented in B (red) and shows that 95% of GFP-positive cells have high p90RSK expression. Forty-eight hours after transfection, cell migration analysis was performed on GFP-positive or -negative cells, unstimulated (Ctrl) or treated with EGF (EGF, 10 nM), UTP (100 µM UTP) or both (EGF/UTP). Data are presented in C and expressed as the mean cell velocity obtained from several independent experiments. Values are means ± s.e.m., $n=3$; *$P<0.001$. (**D**) HaCaT cells were stimulated for the indicated times with 100 µM UTP (UTP), 10 nM EGF (EGF) or both (EGF/UTP). Cell lysates were analysed by western blotting using an anti-phospho-p90RSK (p-p90RSK) antibody as indicated. Immunoblots were then stripped and reprobed with an anti-p90RSK antibody (p90RSK total; upper panel). Histograms show the fold increase of p90RSK phosphorylation normalised to control, untreated cells ($n=3$; *$P<0.05$). (**E**) HaCaT cells were transfected with a myr-p90RSK plasmid (myr-p90RSK) or with an empty plasmid (vector) as described in Materials and Methods. Forty-eight hours after transfection, cells were left unstimulated (–, NT) or stimulated with 10 nM EGF (E) or 100 µM UTP (U) or both (E+U) for 5 min. Cell lysates were analysed by western blotting using anti-p90RSK (p90RSK), anti-β4 integrin (β4) or anti-phospho-p90RSK (p-p90RSK) antibodies. After densitometric analysis, the level of p90RSK phosphorylation was calculated as the ratio of p-p90RSK to β4 integrin (p-p90RSK/β4). The values obtained (lower panel) were expressed as fold increase of control (NT, cells untreated and transfected with vector; $n=3$; *$P<0.05$. (**F**) HaCaT cells were then either left untreated (−BI) or preincubated for 1 h with 10 µM BID-1870 (+BI). As indicated, cells were then stimulated for 45 min with or without 10 nM EGF. HDs were visualised by confocal microscopy using an anti-β4 integrin antibody. Scale bar: 20 µm. Quantification (right) of HD-positive cells was performed as described in Materials and Methods. Values are means ± s.e.m. of three independent experiments (>100 cells per experimental conditions); *$P<0.001$. (**G**) HaCaT cells were transfected to express the active mutant myr-p90RSK (myr-p90RSK) as described in Materials and Methods. Twenty-four hours after transfection, cells were seeded on collagen-I-coated coverslips for 24 h. Cells were then either left unstimulated (Ctrl) or treated for 45 min with EGF (10 nM), UTP (100 µM) or both. HD were visualised by confocal microscopy using an anti-β4 integrin antibody. The level of p90RSK expression was revealed using an anti-p90RSK antibody. Quantification of HD-positive cells was performed as described in Materials and Methods on cells expressing high levels of myr-p90RSK and cells expressing low levels of myr-p90RSK. Values are means ± s.e.m. of three independent experiments (>100 cells per experimental conditions); *$P<0.001$.

(Kobayashi et al., 2006; Giltaire et al., 2011) and other cell types (Ratchford et al., 2010; Erb et al., 2006; Grimm et al., 2010; Liu et al., 2004; Seye et al., 2004) showing that P2Y2R classically activates ERK1/2, like most of the GPCR. Interestingly, similarly to our own observations in HaCaT cells, P2Y receptors activate MAPK pathway in serum-starved astrocytes and exhibit some ability to inhibit this pathway in the presence of growth factors (Lenz et al., 2001). We identified an important distinguishing feature between the two pathways elicited by UTP. Indeed, several reports have shown that ERK1/2 activation by P2Y2R requires cell

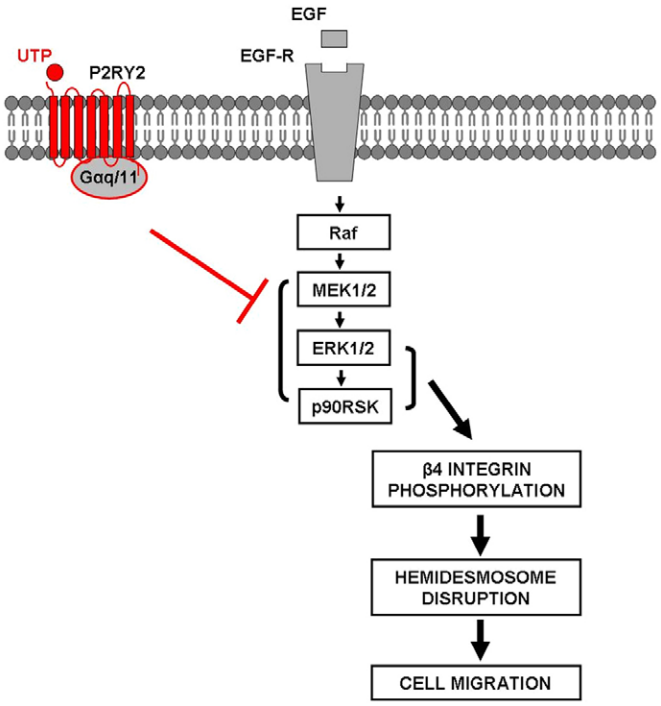

Fig. 8. Proposed model of UTP involvement in HD formation. During wound healing, inflammatory cells, macrophages, endothelial cells and fibroblasts release numerous growth factors, cytokines, chemokines and extracellular nucleotides in the microenvironment. Together, these molecules form a complex signalling network that governs keratinocyte behaviour. This schematic model depicts the crosstalk signalling pathways in HaCat keratinocytes co-stimulated by EGF and UTP. EGF activates EGF receptor (EGFR), Raf–MEK1/2–ERK1/2 signalling pathway and its effector p90RSK to induce β4 integrin phosphorylation (Frijns et al., 2010), and thus HD disruption. HD dismantling participates to the promotion of cell migration. P2Y2 receptor (P2Y2R) and Gαq activation by UTP transduces an unidentified pathway leading to the inhibition of the MAPK signalling cascade downstream of Raf. The resultant inhibition of p90RSK stabilises β4 integrin in HD, a crucial event for HaCat keratinocyte migration inhibition by UTP.

adhesion to substratum (Short et al., 2000; Kudirka et al., 2007) and is improved by the ability of the purinergic receptor to bind to αv integrin via an RGD sequence (Erb et al., 2006; Wang et al., 2005; Kudirka et al., 2007). However, the inhibitory ERK1/2 signalling activity of P2Y2R is anchorage independent (Fig. 2; supplementary material Fig. S2). Thus, P2Y2R triggered a biphasic signal toward ERK1/2, an early one that inhibits most of the EGF-induced ERK1/2 phosphorylation in an adhesion-independent way and a later one that triggers ERK1/2 activation in a cell anchorage-dependent manner. We can assume that P2Y2R receptor signalling through ERK1/2 may be gated by matrix proteins and the state of the relationship between P2Y2R and the integrins may represent a molecular basis for a contextual regulation of P2Y2R function.

Beside purinergic receptors, other GPCR such as adrenomedullin or β2-adrenergic receptors have also been shown to transduce such an inhibitory signal of the ERK pathway (Parameswaran et al., 2000; Pullar et al., 2006). GPCR can antagonize ERK1/2 pathway activation by several mechanisms. For instance, it has long been appreciated that the cAMP produced by Gαs protein and the adenylate cyclase inhibits growth factor-induced ERK1/2 activation (Cook and McCormick, 1993; Wu et al., 1993). To this end, cAMP activates two proteins: the cAMP-dependent protein kinase that directly phosphorylates Raf-1 on inhibitory residues and the GTPase Rap-1 that sequesters Raf-1 away from its activator Ras (Schmitt and Stork, 2002b; Schmitt and Stork, 2002a; Schmitt and Stork, 2001). Another mechanism involving Gα12/13 protein has been showed to directly interact with serine/threonine protein phosphatases 2 and 5 (PP2A and PP5) to transactivate their phosphatase activities (Yamaguchi et al., 2002; Zhu et al, 2007). PP5 mediates Raf-1 dephosphorylation on its activating Ser338 residue (von Kriegsheim et al., 2006) and PP2A acts downstream of Raf (Wassarman et al., 1996). Here, we provide the first experimental evidences indicating that Gαq can transduce ERK1/2 pathway inhibitory signals. We report that UTP is still able to inhibit ERK1/2 phosphorylation induced by an active mutant form of Raf kinase (ΔA:Raf:ER; Fig. 3). Thus, we assume that P2Y2R and Gαq decrease MEK and ERK1/2 phosphorylations downstream of Raf activation by EGF. However, the identification of more precise signalling mechanisms that link P2Y2R/Gαq axis to ERK1/2 pathway requires further investigations.

Since the demonstration that ERK1/2 mediates the EGF-induced keratinocyte motility (Haase et al., 2003), significant advances in the role of ERK1/2 in cell migration regulation have occurred. Indeed, several ERK1/2 targets such as the actin regulators EPLIN, Wave2 and Ab1 have been identified (Han et al., 2007; Mendoza et al., 2011), as well as m-calpain and a RhoA GTPase-activating protein that both regulate the remodelling of actin-based adhesion sites (Webb et al., 2004; Pullikuth and Catling, 2010; Leloup et al., 2010). In addition to these proteins, p90RSK has been identified as a principal effector of ERK1/2 pathway that controls a gene programme leading to the epithelial–mesenchymal transition and to the promotion of epithelial cell migration and invasion (Doehn et al., 2009; Smolen et al., 2010). In this work, we show that pharmacological inhibition of p90RSK strongly reduces the EGF-induced HaCaT cell speed confirming the role of this kinase in the control of cell migration (Fig. 7A). Upon EGFR activation, the destabilization of β4/plectin interaction is driven by the phosphorylation of several residues located in β4 cytodomain including S1424 (Germain et al., 2009), S1356, S1360 and S1364 (Rabinovitz et al., 2004; Wilhelmsen et al., 2007; Frijns et al., 2010) and, more recently discovered, T1736 (Frijns et al., 2012). Interestingly, serines 1364 and 1356 have been identified as potential phosphorylation sites of p90RSK and ERK/1/2. Mutations of these serines to phosphomimicking aspartic acid increase keratinocyte motility. Moreover, in the same study, it was shown that keratinocytes expressing the non-phosphorylatable $\beta4^{S1364A/S1356A}$ integrin are poorly responsive to EGF motogenic stimuli (Frijns et al., 2010). Similar results were obtained on keratinocyte-derived tumours cell lines (Kashyap et al., 2011). In support for a role of ERK1/2 and p90RSK in the regulation of β4/plectin interaction and HD formation (Frijns et al., 2010), we show that the pharmacological inhibition of ERK1/2 and p90RSK increases endogenous β4 integrin localisation in HD upon EGF treatment (Fig. 4, Fig. 7F). Importantly, expression of a p90RSK mutant activated by membrane targeting (Richards et al., 2001) abrogates β4 integrin localisation in HD plaques (Fig. 7G). Together these data emphasise that in keratinocytes β4 stabilisation into HD may be sufficient to inhibit cell migration and that p90RSK activation by ERK1/2 appears to be an essential step in the signalling cascade transduced by EGF leading to HD disruption and cell migration.

The major result of the present study is the discovery that the activation of P2Y2R and Gαq by UTP stabilises HD in two keratinocyte derived cell lines, HaCat and A431 cells. This is revealed by the ability of extracellular nucleotides to impede EGF-induced β4 integrin mobilisation from HD in both cell types (Fig. 5A), as well as plectin, BPAG1, BPAG2 and CD151 in HaCat cells S3, S4). P2Y2R can also promote the formation of these adhesive structures as indicated by the relocalisation of β4 integrin into HD a few minutes after UTP stimulation (Fig. 6). HD stabilisation and formation by the UTP/P2Y2R/Gαq axis is likely a direct consequence of the inhibition of EGF-induced ERK1/2 and p90RSK signalling function. In support of this hypothesis, we observed that in EGF-treated HaCat cells the UTP-induced HD formation (Fig. 6) is timely preceded by UTP-induced ERK1/2 inhibition (Fig. 3C). Moreover, UTP does not inhibit ERK1/2 phosphorylation in MCF10A cells and had no impact on HD dynamics (Fig. 2B, Fig. 5D). A more direct evidence comes from the fact that UTP fails to promote HD formation in HaCaT cells expressing a constitutively activated p90RSK mutant (Richards et al., 2001), which is insensitive to P2Y2R signalling function (Fig. 7E,G).

P2Y2R stabilises HDs and dampens HaCat cell migration over long periods of time (Fig. 1C, Fig. 6; supplementary material Fig. S6). This might be in apparent conflict with the fact that UTP inhibits ERK phosphorylation for a short period of time whereas it activates ERK1/2 at later time points (Fig. 2). However, it should be taken into account that the ERK1/2 cascade is a central signalling pathway, activated by a wide variety of cell surface receptors and regulating a large number of cellular processes sometimes acting in an opposing way. This raised the question of the signal specificity. The extent and amplitude of ERK1/2 activation were the first mechanisms suggested to explain ERK1/2 signal specificity, which can trigger either cell differentiation or proliferation (Marshall, 1995). It should also be noted that the ERK cascade specificity is extended by various scaffolds, anchors, regulators and effectors and by its subcellular compartmentalisation (reviewed in Ebisuya et al., 2005; Caunt et al., 2006). Finally, ERK1/2 signalling pathway is modulated by numerous crosstalks with a large array of signals transduced by many cell surface receptors. Therefore, it is not surprising that, as mentioned above, EGFR and P2Y2R can trigger different cellular processes through ERK1/2 activation. For instance, it has been reported that EGF induces HD remodelling and cell migration whereas P2Y2R-induced ERK1/2 activation can stimulate interleukin-6 release (Yoshida et al., 2006). Thus, careful examination of ERK1/2 cellular localisation or the identification of its binding partners in our experimental settings is required to resolve this question. Furthermore, it would be of interest to determine whether or not UTP is able to inhibit ERK/p90RSK-dependent serine phosphorylation of β4 and if this inhibition is sustained.

This signalling crosstalk between a GPCR and a tyrosine kinase receptor may have an in vivo significance. A local increase in extracellular nucleotide concentration released by inflammatory cells may provide molecular cues to help keratinocytes to form and maintain stable HD and to repress their migration in a microenvironment rich in motogenic growth factors. This may have physiological relevance during the early inflammatory phase of the wound healing that precedes the reepithelialisation phase. Our present data in association with those of the literature show that extracellular nucleotides can act as a double-edged sword in the regulation of cell migration. Indeed, extracellular nucleotides have been reported to either activate or block cell migration in a striking cell-specific manner. These antagonistically regulated responses as their consequences are however not well understood and may lead to either a deleterious (e.g. delaying or favouring a chronic wound healing) or a beneficial (e.g. inhibiting cancer cell invasion and metastasis) process. Its fine-tuning appears to be essential for preserving the organism integrity as the development of safe and effective therapeutic treatments. Thus, dissection of the key signalling parameters that determine the outcome of extracellular nucleotides-P2Y2R stimulation constitutes a major challenge for the future.

Materials and Methods

Cell culture and transfections

Human keratinocyte cell line HaCaT (Boukamp et al., 1988) and human epidermoid carcinoma cell line A-431 were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS; Lonza) in a humidified atmosphere of 5% CO_2 at 37℃. Mammary MCF10A cells were cultured in DMEM/F12 (Lonza, Basel, Switzerland) supplemented with 15 mM HEPES buffer, 5% horse serum, 10 µg/ml insulin, 20 ng/ml EGF, 100 ng/ml choleratoxin and 0.5 µg/ml hydrocortisone, in a humidified atmosphere of 5% CO_2 at 37℃. Stable ΔA-Raf:ER HaCaT cell line was a generous gift of G. Thiel (University of Saarland Medical Center, Homburg, Germany) (Rössler and Thiel, 2004). The activation of ΔA-Raf:ER HaCaT cells was achieved by treatment with 4′-hydroxitamoxifen (4′-OHT; 100 nM, purchased from Sigma-Aldrich). For Gαq silencing, Gαq small interfering RNA (siRNA) nucleofection experiments were performed as previously described (Taboubi et al., 2007) using Amaxa nucleofector according to the manufacturer's protocol (Lonza). For P2Y2R silencing, two pre-designed siRNA targeting to human P2Y2R (siP2Y2R n2 and siP2Y2R n6) and siRNA control (AllStars Neg. siRNA AF) were purchased from Qiagen. Exponentially growing HaCaT cells were transfected with 100 nM of siRNA combined with Oligofectamine agent according to the instructions provided by the manufacturer (Invitrogen). Plasmid pRK7-Myr-avRSK1 was kindly given by J. Blenis (Harvard Medical School, Boston, USA) (Richards et al., 2001) and used to express a membrane-targeted form of p90RSK (myr-p90RSK) in HaCaT cells. The cells were transfected with pRK7-Myr-avRSK1 using Lipofectamine reagent (Invitrogen) according to the instructions provided by the manufacturer.

Antibodies and others reagents

For immunoblotting, rabbit polyclonal antibodies against EGF receptor (EGF-R), phospho-EGF receptor (Tyr845, Tyr992, Tyr1045 or Tyr1068), Elk-1, phospho-Elk-1, RSK1/RSK2/RSK3 and rabbit monoclonal antibodies against phospho-MEK1/2, phospho-p44/42 MAPK (p-ERK1/2), p44/42 MAPK (ERK1/2), phospho-p90RSK, p90RSK, phospho-c-Raf, were purchased from Cell Signaling Technology. The mouse monoclonal antibody against MEK1/2 used for immunoblots was also purchased from Cell Signaling Technology. Anti-β4 mouse monoclonal antibody 450-11 used in western blot was a generous gift from R. Falcioni (Regina Elena Cancer Institute, Rome, Italy) (Kennel et al., 1990). Mouse antibody against tubulin and goat polyclonal anti-Gαq/11 antibody were purchased from Sigma-Aldrich and Santa Cruz Biotechnology, respectively. The expression of Raf-1 was revealed using a mouse monoclonal antibody from Transduction Laboratories (BD). Anti-rabbit HRP-conjugated and anti-mouse HRP-conjugated secondary antibodies were purchased from GE Healthcare Life Sciences.

Used for immunofluorescence, rat-anti-α6 integrin (GOH3) antibody was purchased from eBioscience, mouse anti-plectin antibody from Santa Cruz Biotechnology, mouse monoclonal antibody TS151 against CD151 was a generous gift from E. Rubinstein (INSERM U1004, Villejuif, France) (Serru et al., 1999), mouse monoclonal antibody AA3 against β4 integrin was a generous gift from V. Quaranta (Vanderbilt University Medical Center, Nashville, USA) (Tamura et al., 1990), rabbit monoclonal antibody against γ-2 chain of laminin-332 was a generous gift from P. Simon-Assmann (Zboralski et al., 2010) (INSERM U682, Strasbourg, France), mouse monoclonal antibody against BP180 [anti-collagen XVII (NC16A-3)] was purchased from Abcam and mouse monoclonal antibody against BP230 was purchased from 2B Scientific Ltd. Used for FACS analysis, mouse monoclonal antibody against β4 integrin (clone 3E1) was purchased from Millipore. Anti-mouse Alexa-Fluor-488-conjugated secondary antibodies were purchased from Invitrogen, and anti-rat DylightTM-549- and anti-rabbit DylightTM-549-conjugated antibodies were purchased from Jackson Immunotech.

Pharmacological inhibitors against MEK1/2 (U0126 and PD98059) and Raf-1 (Raf-1 inhibitor) were purchased from MERCK Biosciences. The pharmacological inhibitor of Gαq, YM-254890 was a generous gift from J. Takasaki (Astellas Pharma Inc., Ibaraki, Japan) (Takasaki et al., 2004) and BID-1870 (p90RSK inhibitor) was obtained from the University of Dundee (Sapkota et al., 2007). EGF and UTP were obtained respectively from PEPROTECH and MERCK Biosciences.

Migration assays

Cells were serum-starved overnight, trypsinised, and then seeded (10,000 cells/cm^2) on a laminin-332-enriched matrix as previously described (Taboubi et al., 2007). Cells were incubated for 2 h before the beginning of time lapse recording to allow cell adhesion and spreading. Migration was then analysed using an inverted Nikon microscope at 10× magnification (Melville, NY). Two fields per well were imaged and followed every 6 min over specific indicated times with a Coolsnap HQ camera (Photometrics, Tucson, AZ) operated by NIS elements AR 2.30 software (Nikon). Manual single-cell tracking was performed using Metamorph software (Molecular Devices) as described previously (Sadok et al., 2008). Migration parameters calculated from each individual cell were determined from time-lapse movies.

Immunoblotting

Keratinocytes or MCF10A were seeded for 5 h (100,000 cells per cm^2), serum starved overnight, and then treated as indicated in figure legends. They were lysed on ice in a buffer containing 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Triton X-100, 0.1% sodium deoxycholate, 4 mM EDTA, 50 mM NaF, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM PMSF, 1 µg/ml leupeptin, and 1 µg/ml aprotinin for 20 min at 4℃. Cell lysates were centrifuged for 10 min at 10,000 ***g*** to eliminate cell debris. Equal amounts of protein (Protein Assay Kit, Bio-Rad, Hercules, CA) were separated by SDS-PAGE and then transferred to Hybond-C nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were probed with the appropriate primary antibody (2 µg/ml) and then with a peroxidase-conjugated secondary antibody. Bound immunocomplexes were detected using the enhanced chemiluminescence detection system from Amersham Pharmacia Biotech. Western blots were developed using chemiluminescence detection system in a luminescent image analyser, the G:Box Chemi XT^4 (Ozyme, France). This analyser ensures that the intensity of all bands is in the linear range. Images obtained were analysed using Image J software to quantify the band intensities. Alternatively, following chemiluminescence detection, western blots were developed using autoradiography and the scanned images were quantified using ImageJ software. Densitometric analyses were performed on images obtained after different exposure times to select only the non-saturating pictures.

Immunofluorescence

HaCaT or MCF10A cells were seeded on collagen-I-coated coverslips for 24 h (20,000 cells per cm^2, 10 µg/ml of collagen) and serum starved overnight. As shown in supplementary material Fig. S6, HaCat cells secreted lm-332 and formed HD-like structure on this autologous ECM. A431 cells were plated in DMEM supplemented with 0.1% bovine serum albumin (BSA/Euromedex) for 2 h on collagen-I-coated coverslips (30,000 cells per cm^2, 20 µg/ml of collagen). Then, cells were treated as indicated in the figure legends and then fixed in 3.7% formaldehyde, permeabilised with 0.2% Triton and blocked with 3% bovine serum albumin (BSA)/10% fetal calf serum (FCS). Cells were incubated overnight at 4℃ with the primary antibody and then with the appropriate fluorescent secondary antibody. Cells were observed under immersion oil 63×/1.4 Pan Apochromat objectives on a confocal Leica SP5 microscope (Leica Microsystems).

A quantitative analysis was performed by counting the number of cells showing hemidesmosomal plaques as a percentage of the total number of cells. Hemidesmosomal plaques are visualised as 'leopard spots' where β4 integrin, plectin, BPAG1, BPAG2 colocalise. 'HD-positive' cells are identified by characteristic 'leopard-skin' HD pattern of β4 integrin staining or other HD components. For more details about this quantification, see Figs 4 and 5. This quantitative analysis was performed as 'double-blind' test and for statistical relevance; at least 100 cells were counted for each condition in three to five independent experiments

RNA isolation and qRT-PCR

Total RNA was extracted from HaCaT cells 24 h after their transfection using High Pure RNA Isolation Kit (Roche). cDNA synthesis was performed using the M-MluV RT from Fermentas, in the conditions recommended by the manufacturer. Gene expression was quantified by real time PCR using Quantitect Sybergreen dye (Qiagen) and specific primers against P2Y2R and β2 microglobuline genes (Qiagen). Real-time PCR reactions were carried out on LightCycler 480 (Roche). Beta2 microglobuline was used as endogenous control in the ΔΔCt analysis. We presented efficiency of each siRNAs in supplementary material Fig. S1.

Flow cytometry analysis (FACS)

Untreated or treated HaCaT cells were cultured in serum-free DMEM medium as described above and then incubated for 90 min at 4℃ with antibodies against β4 integrin or with irrelevant control antibody. Washed cells were incubated with FITC-conjugated antibody for 30 min at 4℃, washed, fixed in 2% formaldehyde and subjected to flow cytometry. The relative fluorescence intensity was compared with the fluorescence intensity of the same cells stained with the control antibody. Results were presented as the number of cells versus the log of fluorescence intensity.

Statistical methods

Results are presented as the means ± s.e.m. unless otherwise indicated. Comparison of parameters between treatments conditions was performed using the non-parametric Mann–Whitney test. Results were considered significant at $P<0.05$.

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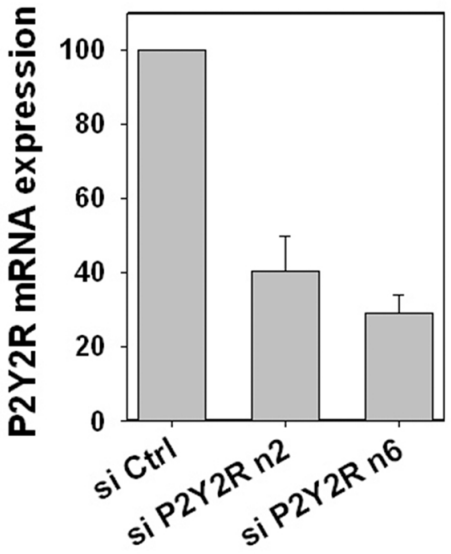


Fig. S1. Analysis of the efficiency of two distinct siRNA against P2RY2 by quantitative reverse transcription polymerase chain reaction (qRT-PCR). qRT-PCR was performed after siRNA transfection on HaCaT cells as described in Materials and Methods. For each experiment, siRNA efficiency was assessed by qRT-PCR. The mRNA levels are expressed as a percentage of the control cells. Data are the mean of 7 independent experiments ± s.e.m.

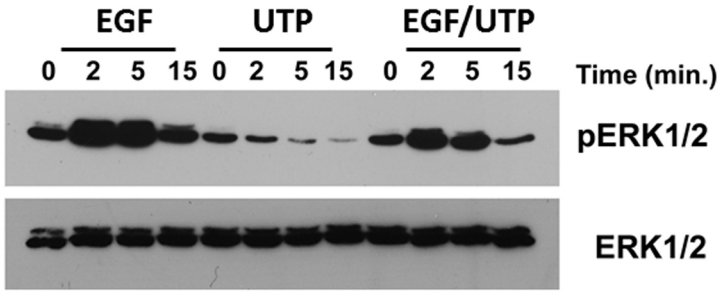


Fig. S2. UTP inhibits EGF-induced ERK1/2 activation in HaCaT cells maintained in suspension. Serum-starved HaCaT were kept in suspension in serum-free medium. Cells were stimulated for the indicated times with 10 nM EGF, 100 µM UTP or both (EGF/UTP). Cell lysates were analysed by western blotting using an anti-phospho-ERK1/2 (p-ERK1/2) antibody. Immunoblots were then stripped and reprobed with an anti-ERK1/2 (ERK1/2) antibody, as indicated.

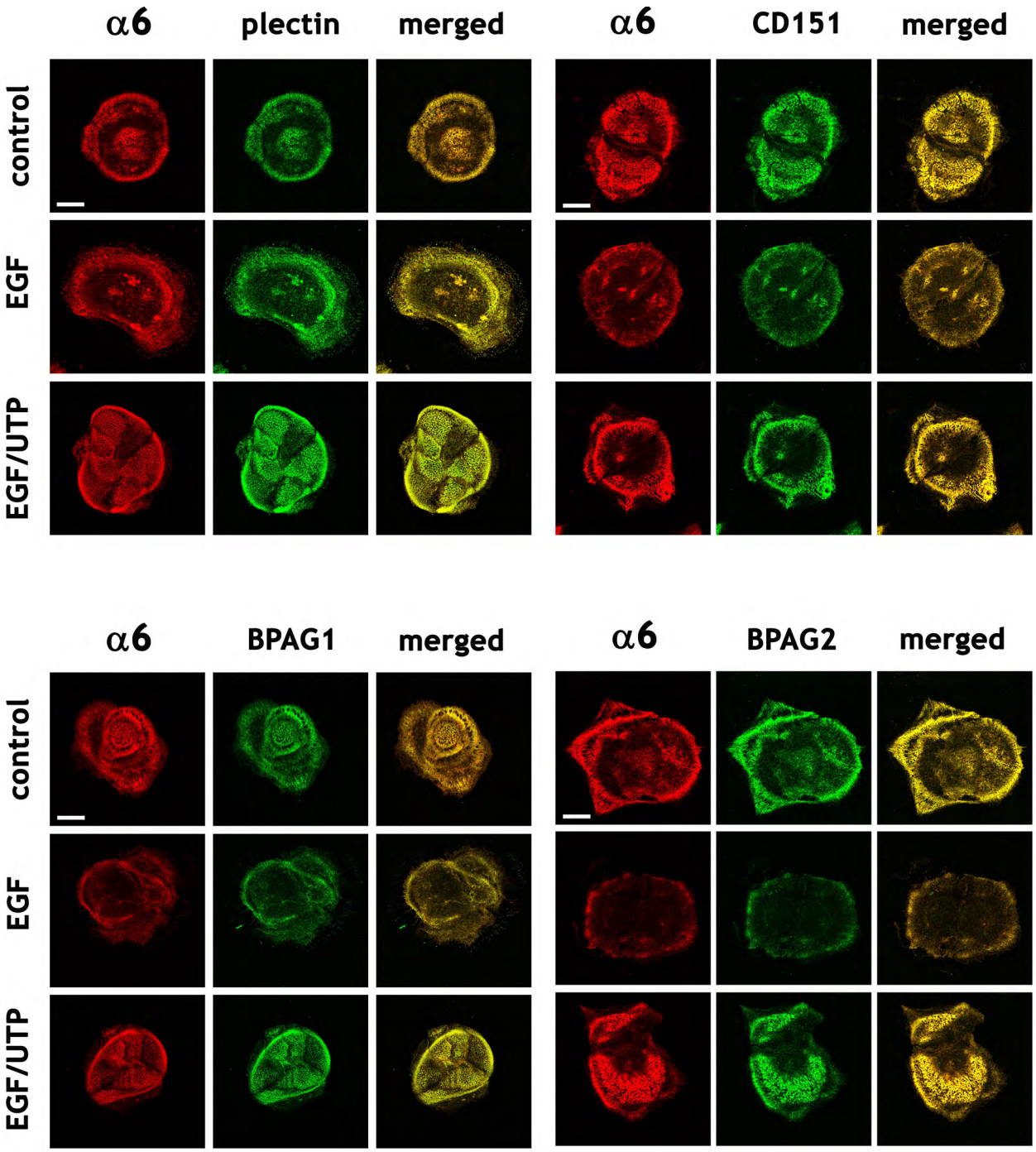


Fig. S3. UTP inhibits the relocalisation of the HD components (α6 integrin subunit, plectin, CD151, BPAG1 and BPAG2). HaCaT cells were seeded on collagen I-coated coverslips for 48h. Cells were then either left untreated (control) or stimulated for 45 min with 10 nM EGF, alone or in the presence of 100 μM UTP (EGF/UTP). HDs were visualized by confocal immunofluorescence using anti-α6 integrin subunit (α6), anti-HD1/plectin (plectin), anti-CD151, anti-BPAG1 and anti-BPAG2 antibodies. Scale bar: 20 μm.

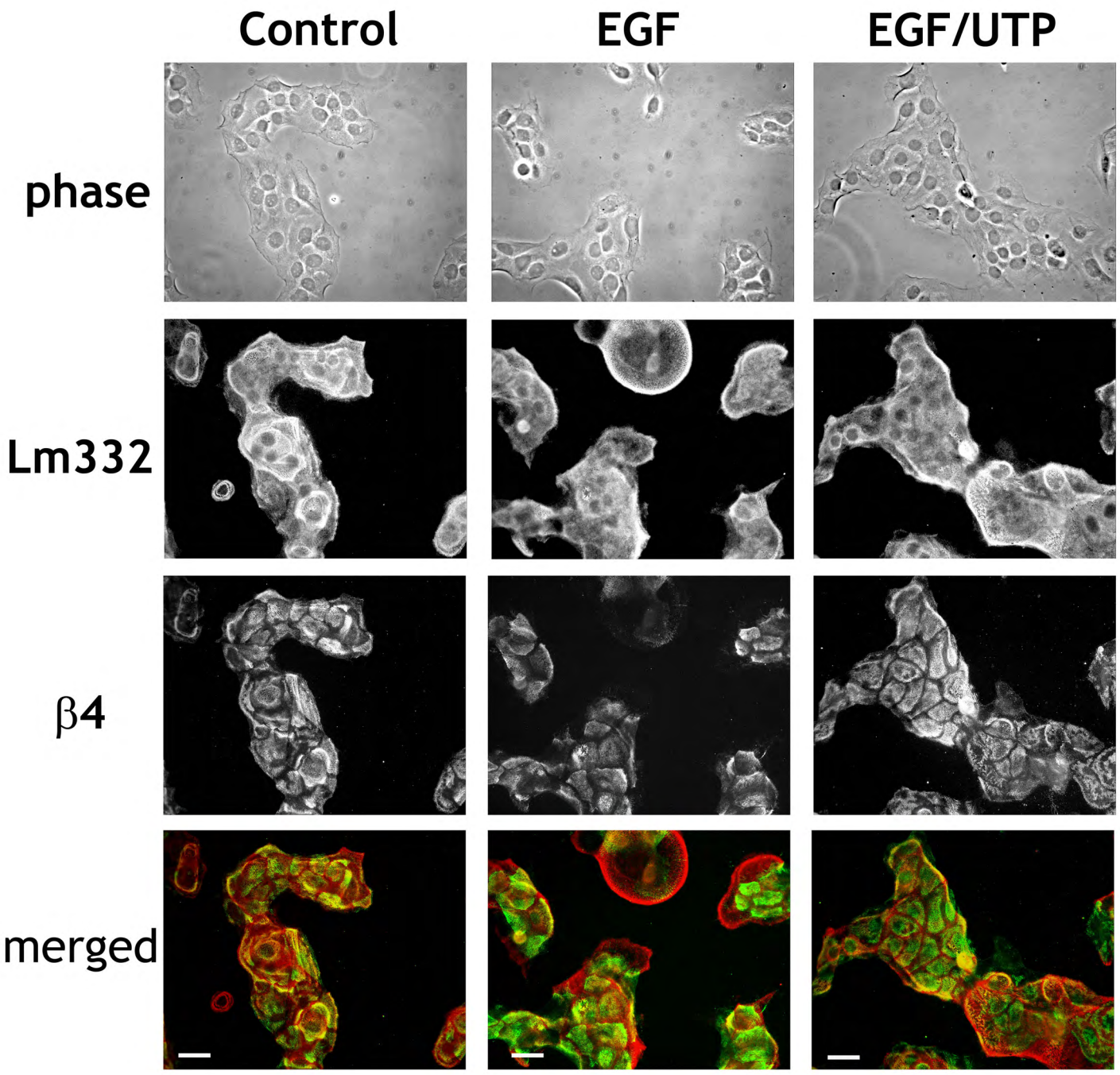
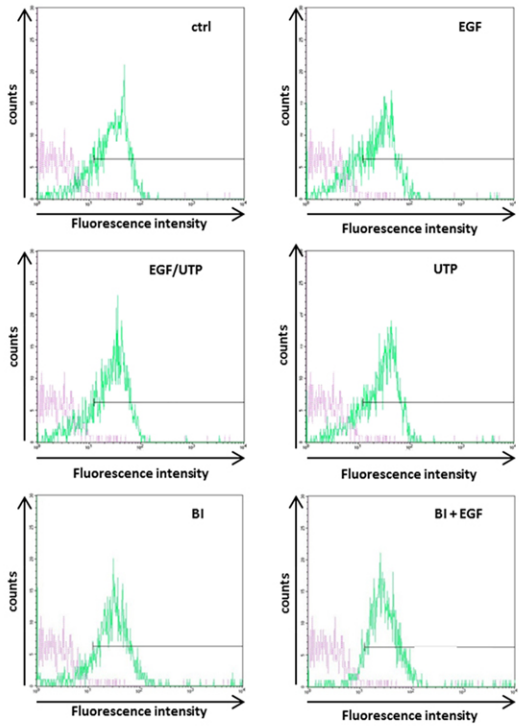


Fig. S4. EGF and UTP have no impact on laminin-332 deposition. HaCaT cells were seeded on collagen I-coated coverslips. Cells were then either left untreated (control) or stimulated for 45 min with 10 nM EGF, alone or in the presence of 100 μM UTP (EGF/UTP). HDs were visualized by wide field immunofluorescence using anti-β4 integrin subunit (β4) and anti-γ2 chain (lm332) antibodies. Scale bar: 50 μm. Note that HaCat form HD on autologous secreted laminin-332 and that EGF and UTP had no effect on lm332 distribution.



treatment conditions	β4 integrin positive cells (mean fluorescence % ± SD)
ctrl	38,5 ± 0,7
EGF	47,5 ± 8,3
EGF/UTP	49,8 ± 10,1
UTP	43 ± 4
BI	49 ± 7,6
BI + EGF	51 ± 7,8

Fig. S5. β4 expression was not changed by EGF or UTP signalling. HaCaT cells were either left untreated (ctrl) or pretreated for 1h with 10 μM BID-1870 (BI). Untreated cells were then stimulated with EGF (10 nM) and UTP (100 μM), alone or in combination (EGF/UTP) for 60 minutes. As indicated, cells pretreated with BID-1870 (BI) were stimulated with or without 10 nM EGF for 60 minutes. Cells were then incubated with antibody against β4 integrin or with irrelevant control antibody and analysed by FACS. The relative fluorescence intensity (green line) was compared with the fluorescence intensity of the same cells stained with the control antibody (pink line). Results are presented as the number of cells versus the log of fluorescence intensity (upper panel). The table shows mean values (%) of relative fluorescence intensity.

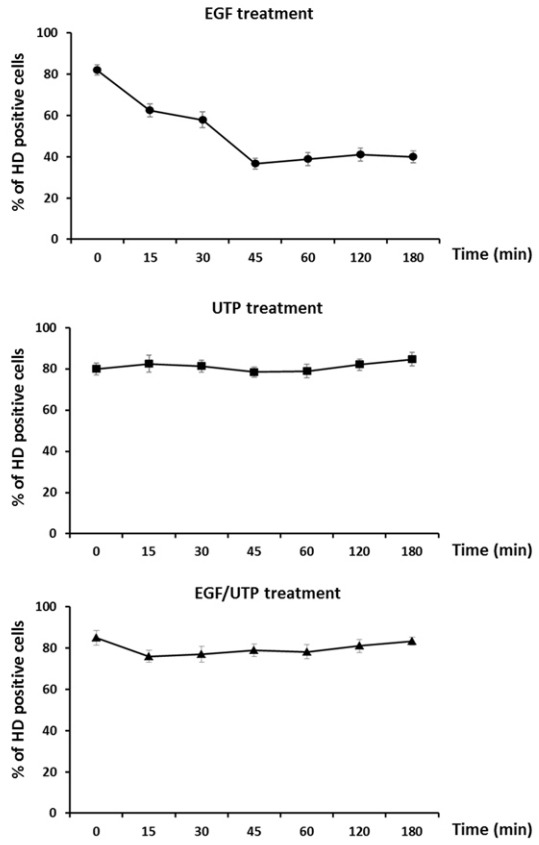


Fig. S6. Time course analysis of EGF and UTP effect on β4 distribution in HD-like plaques. HaCaT cells seeded on collagen I-coated coverslips were stimulated for indicated period of times with either EGF (10 nM), UTP (100 μM) or both (EGF/UTP). HD were visualized by confocal immunofluorescence using anti-β4 integrin subunit (β4). The quantifications of HD positive cells were performed as described in Materials and Methods and are presented in this figure.