A new model system identifies EGFR/HER2 and HER2/HER3 heterodimers as potent inducers of oesophageal epithelial cell invasion

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Abstract

Oesophageal squamous cell carcinomas and oesophageal adenocarcinomas display distinct patterns of ErbB expression and dimers. The functional effects of specific ErbB homo- or heterodimers on oesophageal (cancer) cell behaviour, particularly invasion of early

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carcinogenesis remains unknown. Here, a new cellular model system for controlled activation of EGFR or HER2 and EGFR/HER2 or HER2/HER3 homo- and heterodimers was studied in non-neoplastic squamous oesophageal epithelial Het-1A cells.

EGFR, HER2 and HER3 intracellular domains (ICDs) were fused to dimerization domains (DmrA / DmrA and DmrC), and transduced into Het-1A cells lacking ErbB expression. Dimerization of EGFR, HER2 or EGFR/HER2, HER2/HER3 ICDs was induced by synthetic ligands (A/A or A/C dimerizers). This was accompanied by phosphorylation of the respective EGFR, HER2 and HER3 ICDs and activation of distinct down-stream signalling pathways, such as PLCγ1, Akt, STAT and Src family kinases. Phenotypically, ErbB homo- and heterodimers caused cell rounding and non-apoptotic blebbing in EGFR/HER2 and HER2/HER3 heterodimer cells. In a Transwell assay, cell migration velocity was elevated in HER2-dimer as compared to empty vector cells. In addition, HER2-dimer cells showed in increased cell invasion, reaching significance for induced HER2/HER3 heterodimers ($p=0.015$). Importantly, in three-dimensional organotypic cultures, empty vector cells grew as a superficial cell layer, resembling oesophageal squamous epithelium. In contrast, induced HER2-dimer cells (HER2 homodimers) were highly invasive into the matrix and formed cell clusters. This was associated with partial loss of CK7 (when HER2 homodimers were modelled) and p63 (when EGFR/HER2 heterodimers were modelled), which suggests a change or loss of squamous cell differentiation.

Controlled activation of specific EGFR, HER2 and HER3 homo- and heterodimers caused oesophageal squamous epithelial cell migration and/or invasion, especially in a three dimensional microenvironment, thereby functionally identifying ErbB homo- and heterodimers as important drivers of oesophageal carcinogenesis.

Keywords: Oesophageal cancer, ErbB homo-/heterodimers, non-apoptotic blebbing, cell migration, cell invasion

Introduction

The ErbB family of receptor tyrosine kinases consists of EGFR, HER2, HER3 and HER4 and can be activated by the EGF family of growth factors [1-4]. Upon growth factor binding, the receptors homo- or hetero-dimerize, activate multiple signalling pathways, and guide subsequent cell behaviour [1-4]. Since somatic or cancer cells can express more than one ErbB receptor or ligand, receptor activation results in a complex signalling network [1-4]. This complicates the understanding of how one specific homo- or hetero-dimer contributes to cell signalling, cell behaviour or potential malignant transformation.

In both histotypes of oesophageal cancer, oesophageal squamous cell carcinoma (ESCC) and oesophageal adenocarcinoma (EAC)[5], ErbB gene amplification or overexpression is seen [5-11]. EGFR overexpression is often found in ESCCs, whereas HER2 and/or HER3 are overexpressed in EACs [5-11]. Accordingly, EGFR homodimers are present in ESCCs and HER2 homo- and HER2/HER3 heterodimers occur in EACs [8]. However, the precise contribution of specific ErbB dimers to oesophageal cancer cell behaviour, carcinogenesis or cancer progression is not understood.

In general, ErbB homodimers are less mitogenic and transforming than the corresponding heterodimers [2]. HER2-containing heterodimers are considered most potent, especially HER2/HER3 heterodimers [2]. This was shown in a model of growth arrested mammary acini, which rely on ductal/glandular epithelial cells: HER2 homodimers reinitiated cell proliferation and disrupted cell polarity [12], but EGFR/HER2 heterodimers even induced invasion [13]. These models therefore contribute to the understanding of adenocarcinomas arising from their precursor ductal epithelial cells. Since the output of the ErbB signalling network depends on the cellular origin [1-4], the functional consequences of specific ErbB homo- or heterodimers on oesophageal squamous epithelial cells in terms of signalling and cell behaviour, especially cell morphology (squamous or glandular/intestinal) and cell migration and invasion as early carcinogenesis of either ESCC or EAC is so far unknown.

In previous studies, forced EGFR overexpression by oesophageal epithelial cells indicated that EGFR is important for cell proliferation, migration or epithelial-mesenchymal transition (EMT) [14-16]. In contrast, we and others reported that inhibition of EGFR and/or HER2 in ESCC or EAC cells by tyrosine kinase inhibitors (TKIs) reduced cell proliferation and induced apoptosis [8,17-19]. Moreover, we showed that TKIs also inhibited oesophageal cancer cell migration [20].

However, studies investigating the effects of specific EGFR, HER2 or HER3 dimerization for oesophageal squamous epithelial cells are lacking. Hence, the precise role of ErbB homo- or heterodimers for oesophageal (cancer) squamous cell behaviour, including cell proliferation, migration or invasion, and thereby to oesophageal carcinogenesis or progression is poorly understood. Furthermore, it is unclear whether distinct ErbB expression or dimerization patterns observed in ESCC and EAC are involved in early carcinogenesis or metastasis.

Here, we addressed this question for the first time by using non-neoplastic squamous oesophageal epithelial Het-1A cells [8], which lack endogenous ErbB (over-)expression and dimers, for transduction with constructs containing EGFR, HER2 or HER3 intracellular domains (ICDs) connected to dimerization domains DmrA or DmrC. DmrA is based on the human protein FKBP12, a target for FK506 and rapamycin. DmrB is a mutated 93 amino acid portion of the PI3K homolog FRAP. Rapamycin functions by binding to FKBP and to FRAP, thereby joining the two proteins as a heterodimerizer [13]. These Dmr domains can hence be utilised to induce dimerization of the ErbB-Dmr containing chimaeric proteins by synthetic ligands, respective analogues of rapamycin. As such, the commercial synthetic ligands known as A/A Homodimerizer and A/C Heterodimerizer dimerize DmrA and DmrB, whilst not being able to bind to wild-type endogenous FRAP [21,22]. This allows controlled homodimerization of EGFR or HER2 and heterodimerization of EGFR/HER2 or HER2/HER3 by A/A Homodimerizer and A/C Heterodimerizer treatments.

Hence, this cellular model system enabled us to precisely study the effect of specific ErbB dimers on signalling pathways and oesophageal cell behaviour, such as cell morphology, migration and invasion. Moreover, by using three-dimensional organotypic cultures of the transduced oesophageal squamous epithelial cells, the potential of malignant transformation was studied in terms of histopathological assessment of cell morphology, differentiation and cell invasion into deeper organotypic culture matrix. In an exploratory approach, OTCs showing the most significant phenotype differences were further examined using matrix-assisted laser desorption/ionization Fourier-transform ion cyclotron resonance mass spectrometry imaging (MALDI-FT-ICR MSI) for metabolomic profiling [23,24].

Materials and Methods

DNA constructs

Details of DNA construct preparation are provided in supplementary materials, Supplementary materials and methods. After each cloning step vectors were subjected to sequencing to verify the nucleotide sequences.

Lentiviral stocks

Lentiviral stocks were prepared by using the Lenti-X™ HTX Packaging System with Lenti-X™ 293T cells (Clontech, Takara Bio Europe SAS, Saint-Germain-en-Laye, France) according to the manufacturer's protocols. Lentiviruses were harvested 48 and 72 hours after transfection, concentrated using Lenti-X™ Concentrator (Clontech), titrated using Lenti-X™ p24 RapidTiter Kit (Clontech) according to the manufacturer's protocols and stored at -80 °C.

Cell culture and stable cell lines

Het-1A cells (ATCC, LGC Standards GmbH, Wesel, Germany; non-neoplastic oesophageal squamous epithelial cells, lacking ErbB expression or dimers) [8] were cultured as described

[8]. Authentication was by STR DNA typing [25] (Leibniz Institute DSMZ, Braunschweig Germany). Cells were negative for mitochondrial DNA sequences from rodent cells (mouse, rat, Chinese and Syrian hamster species). Stable Het-1A cells expressing ErbB ICD fusion proteins were generated and controlled for absence of Mycoplasma contamination as described in detail in supplementary material, Supplementary materials and methods.

To activate ErbB dimerisation, 300 nM A/A Homodimerizer (Clontech) in ethanol or ethanol as control (final concentration of ethanol 0.06%) was used for H-e.v.-A/A, H-H1/1 and H-H2/2 cells, whereas 300 nM A/C Heterodimerizer (Clontech) in ethanol or ethanol as control (final concentration of ethanol 0.06%) was used for H-e.v.-A/C, H-H1/2 and H-H2/3 cells.

Indirect immunofluorescence

Indirect immunofluorescence was performed as described [8,20,26] with anti-HA-Tag and anti-Flag-Tag antibodies (supplementary material, Table S1).

Western Blot

Protein lysates and Western Blots were prepared as described [8] and details of all antibodies used are provided in supplementary material, Table S1. Entire Western blots (up to 250 kDa) for phosphorylation status of transduced ErbB ICD fusion proteins and endogenous ErbB proteins are provided in supplementary material, Figures S3 and S4.

Antibody Array

Cells were serum starved (medium + 0.5% FBS) for 24 h. Medium was replaced by starvation medium containing ethanol as a control, 300 nM A/A Homodimerizer in ethanol or 300nM A/C Heterodimerizer in ethanol for 24 h then a Proteom Profiler® Human Phospho-Kinase Array (R&D Systems, Abingdon, United Kingdom) was used according to the manufacturer's

instruction. Quantification of pixel density of duplicate spots was performed using ImageJ 1.47v software in two independent experiments. See also supplementary material, Table S2.

Phalloidin staining and evaluation of blebbing cells

Phalloidin staining was performed as described [20]. Cells were serum starved (medium + 0.5% FBS) for 24 h and medium was replaced by starvation medium containing ethanol as a control, 300 nM A/A Homodimerizer in ethanol or 300nM A/C Heterodimerizer in ethanol for 24 h. In three independent experiments ≥100 cells were randomly captured and evaluated for the occurrence of blebbing cells (blebbing cells/total cells as %).

Organotypic Culturing (OTC)

OTC was performed with modifications to a published protocol [27]. First, a matrix mainly consisting of Matrigel, collagen and fibroblasts (here fetal oesophageal fibroblasts, FEF3) were cultured in Transwell chambers. Upon dislodging matrices from the insert wall on day 2, the fibroblasts organized and were left to contract the Matrigel-collagen matrix for 5 days, which then resembled connective tissue. Thereafter, transduced cells, prior-stimulated with medium containing ethanol as a control, 300 nM A/A Homodimerizer in ethanol or 300 nM A/C Heterodimerizer in ethanol from day 2 to 5, were plated on top of this matrix. From then on, cells were fed with Epidermalization medium I [27] containing ethanol as a control, 300 nM A/A Homodimerizer in ethanol or 300 nM A/C Heterodimerizer in ethanol until day 17 (ie for two weeks). Thereafter, cells were exposed to an air-liquid interface to trigger epithelial differentiation and fed with Epidermalization medium II [27], containing ethanol as a control, 300 nM A/A Homodimerizer in ethanol or 300 nM A/C Heterodimerizer in ethanol for 5 days. Harvested OTCs were fixed in 10.0% formaldehyde and embedded in paraffin, using routine diagnostic automated tissue processing. H&E staining of OTC sections was performed from each of four grossing levels per harvested OTC using automated systems and

reagents (Dako CoverStainer, Agilent, Hamburg, Germany). Three independent experiments for each three-week round of OTCs were performed.

Immunohistochemistry of OTCs

For immunohistochemistry, 2 µm thick serial sections of one grossing level selected from the initial HE-stained OTCs were deparaffinized, subjected to antigen retrieval and incubated with primary antibodies as described in supplementary material, Table S1. After washing, all reactions were continued using the Dako EnVision™ FLEX+ Visualization System on the Autostainer System (all DakoCytomation, Agilent, Hamburg, Germany). Analysis was according to positivity of cells, not staining intensity.

Statistical analyses

To compare untreated and A/A Homodimerizer or A/C Heterodimerizer treated cells, two-tailed, paired Student's t-tests were performed. A p-value of <0.05 was considered statistically significant.

Further materials and methods are provided in supplementary material, Supplementary materials and methods.

Results

Cellular model system for controlled activation of ErbB homo- and heterodimers

The cellular model system (Figure 1A-F) was based on non-neoplastic squamous oesophageal epithelial cells Het-1A, which express the ErbB receptors and form dimers at negligible levels especially as compared to ESCC or EAC cells overexpressing EGFR or HER2 [8]. These cells were transduced with empty vector controls (Figure 1A,D) or with constructs for intracellular

domains (ICDs) of ErbB receptors (Figure 1B,C,E,F). The constructs lacked extracellular domains to prevent spontaneous or ligand induced dimerization. Myristol groups (Myr) ensured the localization to the inner leaflet of the plasma membrane. The DmrA and DmrC domains allowed controlled dimerization of ICDs by synthetic ligands, which act as homodimerizer (A/A Homodimerizer) or heterodimerizer (A/C Heterodimerizer). These induced DmrA/DmrA or DmrA/DmrC dimers in empty vector control cells (H-e.v.-A/A or H-e.v.-A/C cells; Figure 1A,D) and DmrA- and/or DmrC-linked EGFR/EGFR, HER2/HER2, EGFR/HER2 or HER2/HER3 ICDs dimers in H-H1/1, H-H2/2, H-H1/2 or H-H2/3 cells, respectively (Figure 1B,C,E,F).

Membranous expression of the fusion proteins was detected in these 6 transduced Het-1A cell subclones (supplementary material, Figure S1A-F) and transduced cells with comparable fusion protein levels were selected for further analyses (supplementary material, Figure S1G,H). Inducible dimerization of constructs was validated by proximity ligation assay of A/A Homo- or A/C Heterodimerizer treated empty vector control (H-e.v.-A/A and H-e.v.-A/C cells) and all ErbB-dimer cells (supplementary material, Figure S2A-J).

Activation of ErbB ICDs by corresponding A/A or A/C dimerizers resulted in EGFR or HER2 phosphorylation in H-H1/1 or H-H2/2 cells (Figure 1H,I), EGFR and HER2 phosphorylation in H-H1/2 cells (Figure 1K) or HER2 and HER3 phosphorylation in H-H2/3 cells (Figure 1L). Thereby, ErbB receptor phosphorylation and activation started between 0.5 and 8 h and lasted for, or even increased within, 72 h of treatment (Figure 1H,I,K,L). Only transduced ErbB ICD fusion proteins (~100-150 kDa), and no potential endogenous ErbB (EGFR, HER2, HER3: 170-185 kDa) phosphorylation was detected (shown for 72 h post dimerizer treatment in entire Western blots, supplementary material, Figures S3 and S4). Corresponding empty vector control cells (H-e.v.-A/A, H-e.v.-A/C) expressed stable dimerization domains (Figure 1G,J), whereby corresponding dimerizers did not induce potential endogenous EGFR, HER2 or HER3 (170-185 kDa) phosphorylation, or DmrA or DmrC (15-30 kDa) phosphorylation

(supplementary material, Figures S3A-F, supplementary material, Figure S4A-H, entire Western Blots provided).

Activated ErbB-dimers induce distinct down-stream signalling pathways, but do not increase cell proliferation

To examine signalling pathways associated with ErbB-dimer activation, Human Phospho-Kinase Arrays reflecting site-specific phosphorylation of 38 kinases and 2 related total proteins (ß-catenin, HSP60) at 24 h post dimerizer treatment, as well as additional Western blots for two phosphorylation sites of the EGFR/HER2-PLCγ1 axis [28,29], were performed. ErbB-dimer cells showed general alterations in PI3K/AKT, TOR, PLCγ1, STAT and Src-family kinases of >1.5-fold compared to their paired non-induced cells (supplementary material, Table S2), however this was variable in and in part also observed in empty vector control cells. Following normalization to corresponding non-induced empty vector control cell data, activated EGFR homodimers were seen to have caused STAT6, Src family kinases (Yes, Hck), c-Jun and PLCγ1^{Tyr783} phosphorylation, whereas activated HER2 homodimers induced Akt, variable STAT5 and STAT6 as well as Src family kinase (Yes, Hck, Lyn) phosphorylation (Figure 2A). Activated EGFR/HER2 heterodimers led to STAT5b, STAT6, Src family kinase (Yes, Hck) and JNK phosphorylation, whereas in HER2/HER3 heterodimer cells controlled activation led to JNK phosphorylation, with other proteins being variably phosphorylated (Figure 2B). Except for EGFR/EGFR cells, no marked phosphorylation of PLCγ1 at Tyr783 was seen in induced ErbB-dimer cells as compared to empty vector control cells in this analysis (Figure 2A,B). Western blot analyses of PLCγ1^{Tyr783} and PLCγ1^{Ser1248} at 8 and 24 h post dimerization (Figure 2C) showed some background activation, i.e. presence of both PLCγ1^{Tyr783} and PLCγ1^{Ser1248} already at 0 hours in non-induced empty vector control (H-e.v.-A/A) and ErbB-homodimer (H-H1/1, H-H2/2) cells. Phosphorylation of PLCγ1^{Tyr783} was

increased in induced EGFR/EGFR- (H-H1/1) and HER2/HER2- (H-H2/2) dimer cells at 8 hours and remained stable until 24 hours. Phosphorylation of PLCγ1^{Ser1248} was slightly elevated at 8 h and then returned to background levels at 24 h in empty vector control (H-e.v.-A/A) and ErbB-homodimer (H-H1/1, H-H2/2) cells. In contrast, in empty vector control (H-e.v.-A/C) cells phosphorylation of PLCγ1^{Tyr783} was not detected and was stable (i.e. with background activation at 0 h) in ErbB-heterodimer (H-H1/2, H-H2/3) cells. Phosphorylation of PLCγ1^{Ser1248} was similarly stable, without significant changes upon induction of dimerization in all cells.

Although several of the altered signal proteins are involved in cell proliferation [30-34], no marked effect was seen on cell proliferation, except up-regulation of c*yclin D1* (*CCND1*) mRNA levels in HER2/HER2 (H-H2/2) cells (supplementary material, Figure S5A,B) and slightly elevated cell proliferation in H-e.v.-A/A and HER2/HER2 (H-H2/2) cells (supplementary material, Figure S5C-H).

ErbB dimerization alters cell morphology, cell migration and invasion

As analysed by F-actin staining, empty vector control H-e.v.-A/A and H-e.v.-A/C cells had a widespread cytoplasm with several stress fibres and there were no obvious morphological changes after A/A Homo- or A/C Hetero-dimerizer treatment (Figure 3A,D). Similar morphologies were seen in non-activated EGFR (H-H1/1), HER2 (H-H2/2) homodimer or EGFR/HER2 (H-H1/2) and HER2/HER3 (H-H2/3) heterodimer cells, except for H-H1/2 and H-H2/3 cells presenting with round morphology (Figure 3B,C,E,F, arrows). However, upon homo- or hetero-dimer activation more ErbB cells became round shaped (Figure 3B,C,E,F, arrows) and membrane blebbing occurred as a trend in H-H1/2 and significantly in H-H2/3 ($p=0.041$) cells (Figure 3E-H, arrowheads).

Membrane blebbing may occur during apoptosis, cytokinesis or cell migration [35,36]. Assessment of apoptosis via nuclear condensation or fragmentation or expression of the

caspase cleaved CK18 neo-epitope M30 did not yield signs of apoptosis in blebbing and hence possibly migrating H-H1/2 and H-H2/3 cells (supplementary material, Figure S6B,C), whereas H-e.v.-A/C cells treated with lapatinib as control were clearly apoptotic (supplementary material, Figure S6A).

In a Transwell assay, cell migration or invasion velocity was not affected by A/A Homodimerizer treatment of empty vector (H-e.v.-A/A), EGFR (H-H1/1) or HER2 (H-H2/2) homodimer cells (supplementary material, Figure S6D-I for representative charts and supplementary material, Figure S7A,B for quantification). In contrast, cell migration velocity was significantly increased in activated EGFR/HER2 (H-H1/2; p=0.049) and HER2/HER3 (H-H2/3; p=0.028) heterodimer cells at early time points (0-12 h), approximating each other in non-activated and activated cells at later (12-24 h) time points (supplementary material, Figure S6J-L, supplementary material, Figure S7C). Furthermore, activation of HER2/HER3 heterodimer (H-H2/3) cells increased their cell invasion velocity (0-12 h: p=0.077; 12-24 h: p=0.015) (supplementary material, Figure S6O, supplementary material, Figure S7D).

ErbB homo- and heterodimers induce cell invasion in organotypic cultures

To more closely mimic the *in situ* situation, the influence of ErbB dimers was further studied in three-dimensional organotypic cultures (OTCs) [27].

H-e.v.-A/A cells formed non-invasive squamous epithelial layers and A/A Homodimerizer treatment failed to induce EGFR or HER2 phosphorylation or morphological changes (Figure 4A). H-H1/1 cells were also non-invasive, but presented with a slightly increased epithelial thickness in non-induced and induced H-H1/1 cells. Here, EGFR homodimer activation slightly induced EGFR phosphorylation and invasion of selected cells (Figure 4B). Similarly, also for non-induced and induced H-H2/2 cells the epithelial thickness was generally increased as compared to H-e.v.-A/A cells. Few H-H2/2 cells invaded into the matrix without

treatment, but activation of H-H2/2 cells induced HER2 phosphorylation and deep invasion of cell groups (Figure 4C).

H-e.v.-A/C cells formed non-invasive squamous epithelial layers, with already some thickening (but not necessarily matrix invasion) in both non-treated and A/C Heterodimerizer treated cells (Figure 5A) when compared to H-E.v.-A/A cells (Figure 4A). Thereby, H-e.v.-A/C cells displayed single cells being positive for phospho-HER3. In untreated H-H1/2 cells, the epithelial layer presented even thicker than that of H-e.v.-A/C cells with few phospho-HER2 positive cells invading into the matrix (Figure 5B). However, EGFR/HER2 heterodimer activation in H-H1/2 cells strongly increased EGFR and HER2 phosphorylation resulting in a very thick and pleomorphic epithelial layer with highly invasive cells (Figure 5B). Similarly, non-activated H-H2/3 cells showed some thickening and borderline invasion of the epithelial layer, but this was markedly reinforced by HER2/HER3 heterodimer activation (Figure 5C). Thereby, activation induced more phospho-HER2 and phospho-HER3 positive cells (Figure 5C).

ErbB-dimers alter cell differentiation and metabolomic states in OTCs

Cell proliferation was similarly high in all the ErbB-dimer and empty vector control cells in OTCs (supplementary material, Figure S8A-F), hence excluding dimerization-dependent cell proliferation as basis for the increased epithelial layer thickness in OTCs.

Cell invasion is associated with reduced formation of focal contacts [37,38], but focal adhesion kinase (FAK) expression was prominent in OTCs (supplementary material, Figure S8G-L), except in activated EGFR/HER2 (H-H1/2) OTCs showing fewer FAK-positive cells (supplementary material, Figure S8K).

Therefore, cell differentiation in OTCs was assessed using the routine diagnostic markers p63 and CK7, which are more frequently, but not exclusively expressed in ESCC and EAC, respectively [39-41]. Cdx2, which marks glandular/intestinal differentiation in EACs, was

confirmed to be absent by routine diagnostic immunohistochemistry, which was expected in view of the lack of glands in HE stained OTCs.

In OTCs of all transduced, non-induced cells both p63- or CK7-positive cells were present. No marked changes of p63 or CK7 were detected in induced H-e.v.-A/A, H-H1/1 or H-H2/2 cells (Figure 6A,B,C). However, CK7-positive cells were more frequent and stable in non-activated and activated H-H1/2 and H-H2/3 cells compared to H-e.v.-A/C cells (Figure 6D,E,F). In contrast, p63-positive cells were fewer in activated as compared to non-activated H-H1/2 cells (supplementary material, Figure S6E), suggesting loss of original epithelial differentiation.

Finally, exploratory matrix-assisted laser desorption/ionization Fourier-transform ion cyclotron resonance mass spectrometry imaging (MALDI- FT-ICR MSI) experiments [23,24] were performed using OTCs from non-induced and induced empty vector control (H-e.v.-A/C) and HER2/HER3 (H-H2/3) cells, which exhibited the most altered phenotype. Indeed, the metabolomic profiles also were specifically changed upon induction of HER2/HER3 (supplementary material, Figure S9). Thereby, a majority of metabolites was similarly altered (increase or decrease) in induced versus non-induced cells (H-e.v.-A/C, H-H2/3), most likely reflecting traces of A/C Heterodimerizer action and associated metabolites. However, several metabolites were exclusively detected at high levels in induced HER2/HER3 (H-H2/3) cells.

Discussion

ErbB overexpression or gene amplification occurs in both histotypes of oesophageal cancer [5-11], with preferential EGFR overexpression and homodimers in ESCCs and HER2 overexpression and HER2- homo- or HER2/HER3 heterodimers in EACs [8]. However, the functional consequences of specific ErbB dimers on oesophageal squamous epithelial cell behaviour with respect to early carcinogenesis are still unknown. We here present a new cellular model system for controlled activation of ErbB homo- or heterodimers in non-

neoplastic oesophageal squamous epithelial Het-1A cells, which *per se* lack marked (over-)expression of endogenous ErbB receptors or ErbB-dimers [8], and report that specific ErbB-dimers cause an invasive phenotype in organotypic cultures, mimicking early steps of oesophageal carcinogenesis.

Our model system is based on intracellular domains (ICD) of selected ErbB-family receptors in order to allow controlled – ligand independent – activation. Hence, this may not fully recapitulate the "real world" situation of ErbB-family receptors in oesophageal carcinomas. However, a predominance or even exclusivity of a particular EGFR, HER2 or HER3 expression, dimerization and function was reported in several oesophageal cancer cell lines and oesophageal carcinomas *in situ* [8,20]. Moreover, in the present study mere activation of ErbB-dimers via ICDs was sufficient for an invasive, malignant phenotype in three-dimensional organotypic cultures (OTC). As shown by MALDI- FT-ICR MSI [23,24], not only signalling pathways, but also metabolomic profiles were altered upon HER2/HER3 dimerization of H-H2/3 cells in OTCs, accompanying their invasive phenotype. At the routine histological level, OTCs of EGFR/HER2 and HER2/HER3 cells exhibited a stable high frequency of CK7-positive cells, but a lower frequency of p63-positive cells upon induction of EGFR/HER2 cells. Intestinal/glandular precursor lesions and EACs are mainly CK7 positive [39]. P63 staining is positive in stratified epithelia [40,41] and is lost upon cell migration, invasion and metastasis [40,42-43] as well as upon squamous cell trans-differentiating into intestinal/glandular precursor lesions of EAC [44]. The transcription factor CDX2, which is associated with intestinal/glandular epithelial precursor lesions and EAC [39,44,45], was absent in our OTCs, which at three weeks of culture did not (yet) show overt glands. Hence, the pattern of CK7 and p63 observed in the ErbB-dimer OTCs at three weeks growth – at least partially – appears to reflect early carcinogenesis at which oesophageal squamous epithelial cells become invasive and start to de-differentiate. Thus, mere induction of ErbB-dimer ICDs causes overt phenotypic changes and thereby underlines also other

reports in which ErbB receptors act independent of ligands and/or may even present as extracellular truncated EGFR (or EGFRvIII) or cleaved HER2 (p.95) proteins [46,47].

Our findings using this oesophageal cell model may be compared with studies on growth arrested mammary acini [12,13]. In the latter, mammary (ductal/glandular) cell model, EGFR homodimers did not alter acinar structures [12], whereas HER2 homodimers and especially EGFR/HER2 heterodimers disrupted polarised acinar organisation [12,13] and induced matrix-composition dependent invasive protrusions [13]. Hence, our data underline a general function of ErbB homo- and heterodimers in epithelial cell migration and invasion and underscore their functional impact on oesophageal cell behaviour.

Interestingly, some studies of HER2 structure proposed that it cannot form homodimers [48], which somewhat appears to contradict the above findings in oesophageal and mammary epithelial cell models and carcinomas. However, several studies demonstrated the presence of HER2 homodimers in cancer cell lines and/or human cancer tissue specimens [49-52]. In addition, the extracellular domain of HER2 is reminiscent of the ligand-bound form of other ErbB receptors and HER2 is constitutively extended, exposing its dimerization arm, so that it is constantly primed for interaction [4]. Moreover, other basic research studies reported the molecular and structural architecture of an ErbB2 homodimer [53]. Therefore, the presence of high membranous levels of HER2, occurring in EACs due to gene amplification [11], may result in HER2 homodimer formation, which was also shown in our previous study in oesophageal cancer cell lines and oesophageal carcinomas [8].

In our model system, activation of EGFR, HER2 and HER3 homo- or heterodimers induced distinct patterns of ErbB (ICD) phosphorylation and associated down-stream signalling pathway activation. Thereby, monolayer cultures harvested at early time points (0 to 72 h post dimerization) showed different levels of HER2 and HER3 phosphorylation as compared to cells in organotypic cultures, which needed three weeks for “building”. This may actually

indicate that also in human carcinomas *in situ*, phosphorylation dynamics of ErbB receptors differ from those studied under short time experimental cell line culture settings.

Since also the original Het-1A cells are *per se* highly proliferative, ErbB-dimer induction and phosphorylation had no marked effect on cell proliferation. Only a slight increase in the level of Akt phosphorylation with parallel up-regulation of *CyclinD1* mRNA expression was seen in HER2 homodimer cells. Instead, controlled activation of ErbB homo- and heterodimers resulted in activation of distinct STAT transcription factors, Src-family kinases and PLCγ1, all associated with round cell morphology, non-apoptotic blebbing and (amoeboid) cell migration and invasion [30-38]. Accordingly, cell rounding occurred in almost all induced ErbB-dimer cells, being more evident upon heterodimer activation. Furthermore, EGFR/HER2 and HER2/HER3 dimer activation caused non-apoptotic blebbing, cell migration and invasion. In fact, EGF induced ErbB-dimer associated non-apoptotic blebbing and migration of prostate cancer cells [54]. Cells moving in such an amoeboid manner may be influence by the composition and compactness of the extracellular matrix (ECM) [35-38]. Although this aspect was not further followed functionally *in vitro* or in the organotypic cultures, the phenotype of the invasive ErbB-dimer cells in organotypic cultures underlines these cell movements.

In summary, controlled activation of specific EGFR, HER2 and HER3 homo- and heterodimers caused oesophageal squamous epithelial cell migration and invasion, especially in a three dimensional microenvironment, thereby functionally identifying ErbB homo- and hetero-dimers as important drivers of oesophageal carcinogenesis.

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Author contributions statement

C.D.F. designed the study, carried out experimental work, analysed and interpreted data, prepared figures and tables, drafted and revised the manuscript.

C.M.P. carried out experimental work.

A.B. performed MALDI-MSI experiments and analysed the data

N.H. carried out experimental work and assisted in preparation of figures.

B.R. carried out the experimental work on PLCγ1 protein expression and phosphorylation.

L.S. carried out experimental work.

H.N. provided fetal oesophageal fibroblasts 3, assisted with OTC methodology and approved the manuscript.

A.W. designed MALDI-MSI experiments, analysed and interpreted data, approved manuscript

T.R. assisted in xCELLigence migration and invasion assays and approved the manuscript.

M.W. supervised study, interpreted data and approved the manuscript.

S.L. designed and supervised the study, analysed and interpreted data, co-wrote, revised and approved the manuscript.

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Figure legends:

Figure 1: Novel cellular model system for controlled dimerization and activation of ErbB homo- and heterodimers in oesophageal epithelial cells. (A-F) Schematic representation of the modified intracellular domains (ICDs) of ErbB receptors for controlled activation by the synthetic ligands A/A Homodimerizer and A/C Heterodimerizer. **(A, D)** The empty vector controls H-e.v.-A/A and H-e.v.-A/C contain HA- or Flag-tagged dimerization domains A or C (DmrA, DmrC) and are connected to myristoyl groups (Myr). **(B, C, E, F)** Intracellular domains (ICDs) of EGFR, HER2 and HER3 are located to the inner leaflet of the plasma membrane via myristoyl groups (Myr) and are cloned to HA- or Flag-tagged DmrA or DmrC. **(B, C)** A/A Homodimerizer binds to two DmrA domains and induces homodimerization of EGFR (H-H1/1) or HER2 (H-H2/2) ICDs. **(E, F)** A/C Heterodimerizer binds to DmrA and DmrC and induces heterodimerization of EGFR/HER2 (H-H1/2) and HER2/HER3 (H-H2/3) ICDs. **(G-L)** Western blots of fusion protein levels detected using antibodies against HA- or Flag-Tag in H-e.v.-A/A, H-H1/1, H-H2/2, H-e.v.-A/C, H-H1/2 and H-H2/3 cells. EGFR, HER2 or HER3 phosphorylation status is shown for H-H1/1, H-H2/2, H-H1/2 and H-H2/3 cells treated with 300 nM A/A Homodimerizer in ethanol or A/C Heterodimerizer in ethanol at 0, 0.5, 8, 24, 48 and 72 hours. Blots are representative for three independent biological experiments. Phosphorylation status of H-e.v.-A/A and H-e.v.-A/C cells is shown separately with entire Western Blot images in supplementary material, Figure S3 and supplementary material, Figure S4 to completely show phosphorylation at the level of endogenous ErbB receptors (170-185 kDa), of modified ErbB ICDs of the model system (100-130 kDa), or of dimerization domains of the empty vector controls (15-30 kDa). **(J, K)** The β-Actin loading control is shown twice because the same blot was probed repeatedly.

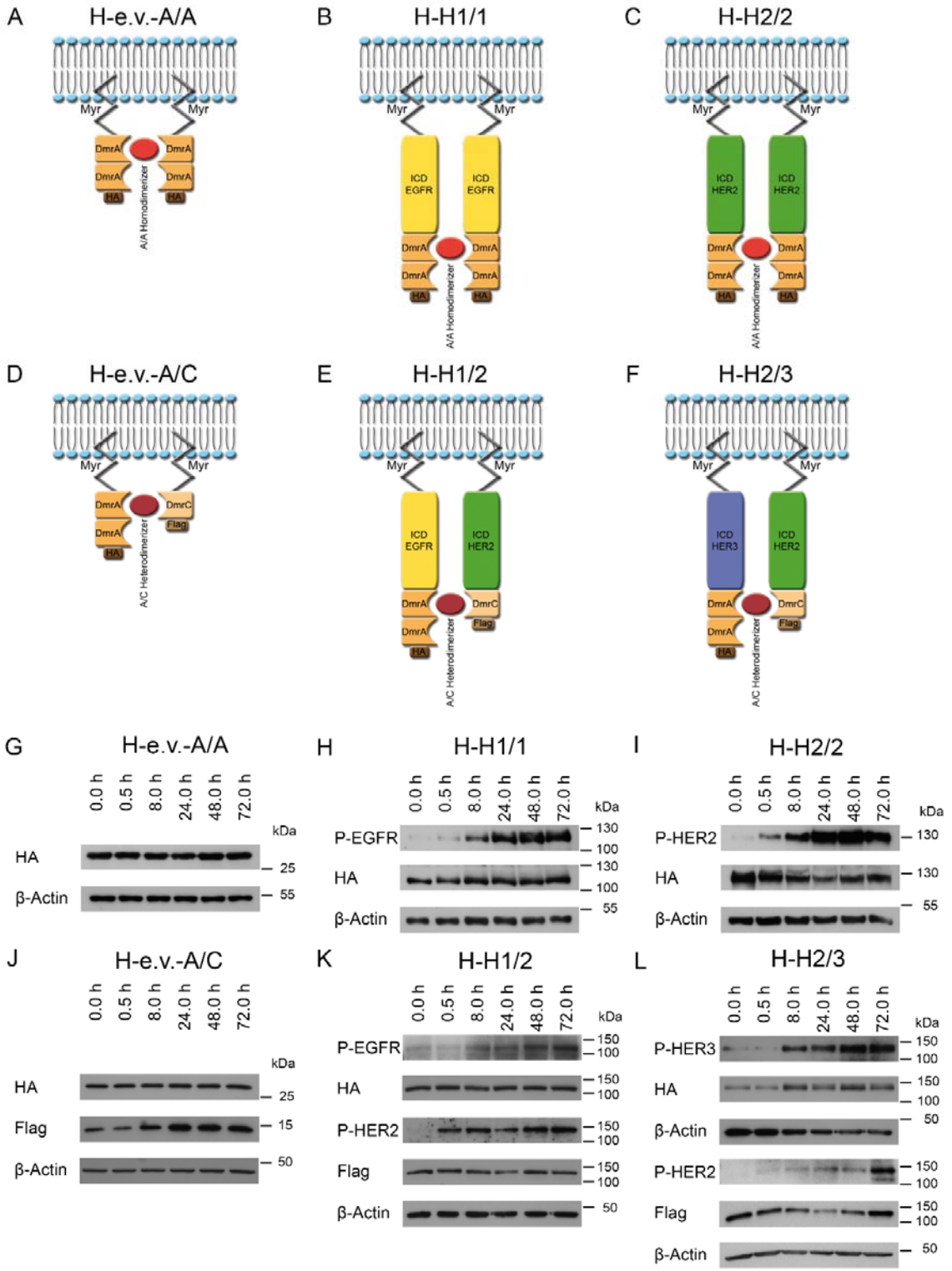


Figure 2: Dimerization induces distinct signalling pathways in H-H1/1, H-H2/2, H-H1/2 and H-H2/3 cells. (A, B) Phospho-kinase array analysis of H-e.v.-A/A, H-H1/1 and H-H2/2 cells (A) as well as of H-e.v.-A/C, H-H1/2 and H-H2/3 cells (B) was performed. Cells were treated with ethanol as a control (-), 300 nM A/A Homodimerizer in ethanol (+) (A) or 300 nM A/C Heterodimerizer in ethanol (+) (B) for 24 h. The graphs present the quantified mean and standard deviation of two independent biological experiments with each two technical replicates as fold-changes relative to the ethanol treated H-e.v.-A/A or H-e.v.-A/C cells. Phosphorylation sites of depicted proteins are given in the graphs in two lanes, with "no add." = only one phosphorylation site measured. Refer to supplementary material, Table S2 for all proteins, respective phosphorylation sites investigated by Human Phospho-Kinase Arrays. **(C)** Western blot analyses for phosphorylation of PLCγ1 at Tyr783 and at Ser1248 of protein lysates at 0, 8 and 24 h post dimerizer treatment. Total PLCγ1 and β-tubulin are shown as loading controls.

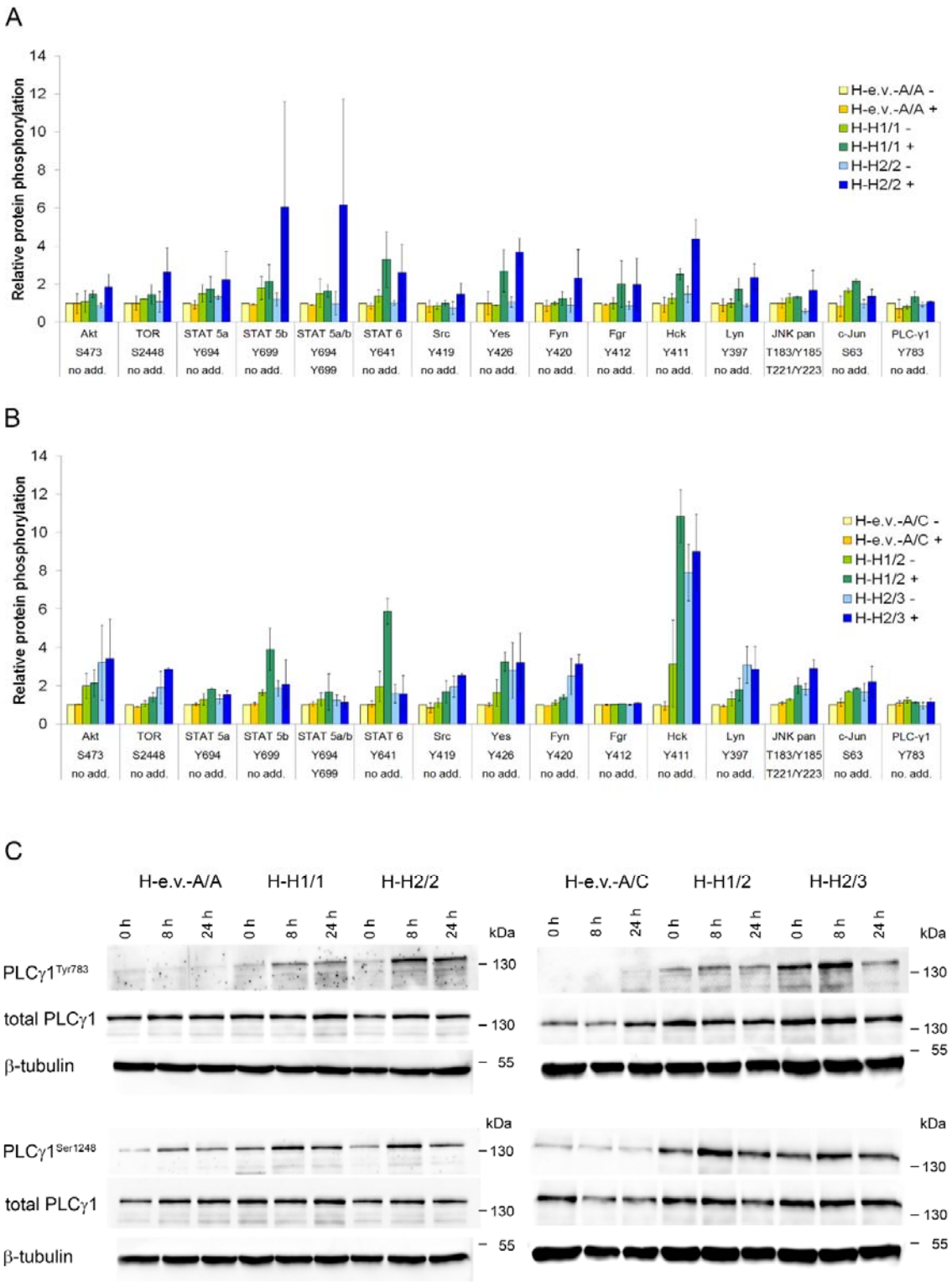


Figure 3: Increased membrane blebbing in H-H1/2 and H-H2/3 cells after A/C Heterodimerizer treatment. (A-F) Representative images of F-Actin staining with fluorescent phalloidin (green) in H-e.v.-A/A **(A)**, H-H1/1 **(B)**, H-H2/2 **(C)**, H-e.v.-A/C **(D)**, H-H1/2 **(E)** and H-H2/3 **(F)** cells treated with ethanol as a control, 300 nM A/A Homodimerizer in ethanol or 300 nM A/C Heterodimerizer in ethanol for 24 h. Cell nuclei were counterstained with DAPI (blue). White arrows indicate rounded cells, whereas white arrowheads indicate membrane blebs. Bar = 20 μm, all panels are at the same magnification. Each inset shows a magnified view (3x) of representative characteristics of the pictured cells. **(G, H)** The bar charts show quantification of cells with membrane blebbing after treatment with ethanol as a control, 300 nM A/A Homodimerizer in ethanol or 300 nM A/C Heterodimerizer in ethanol for 24 h. In total ≥100 cells were counted and the percentage of blebbing cells was determined. Mean and standard deviation of three independent biological experiments are presented. *Significance values from Student's t-test (paired, two-tailed).

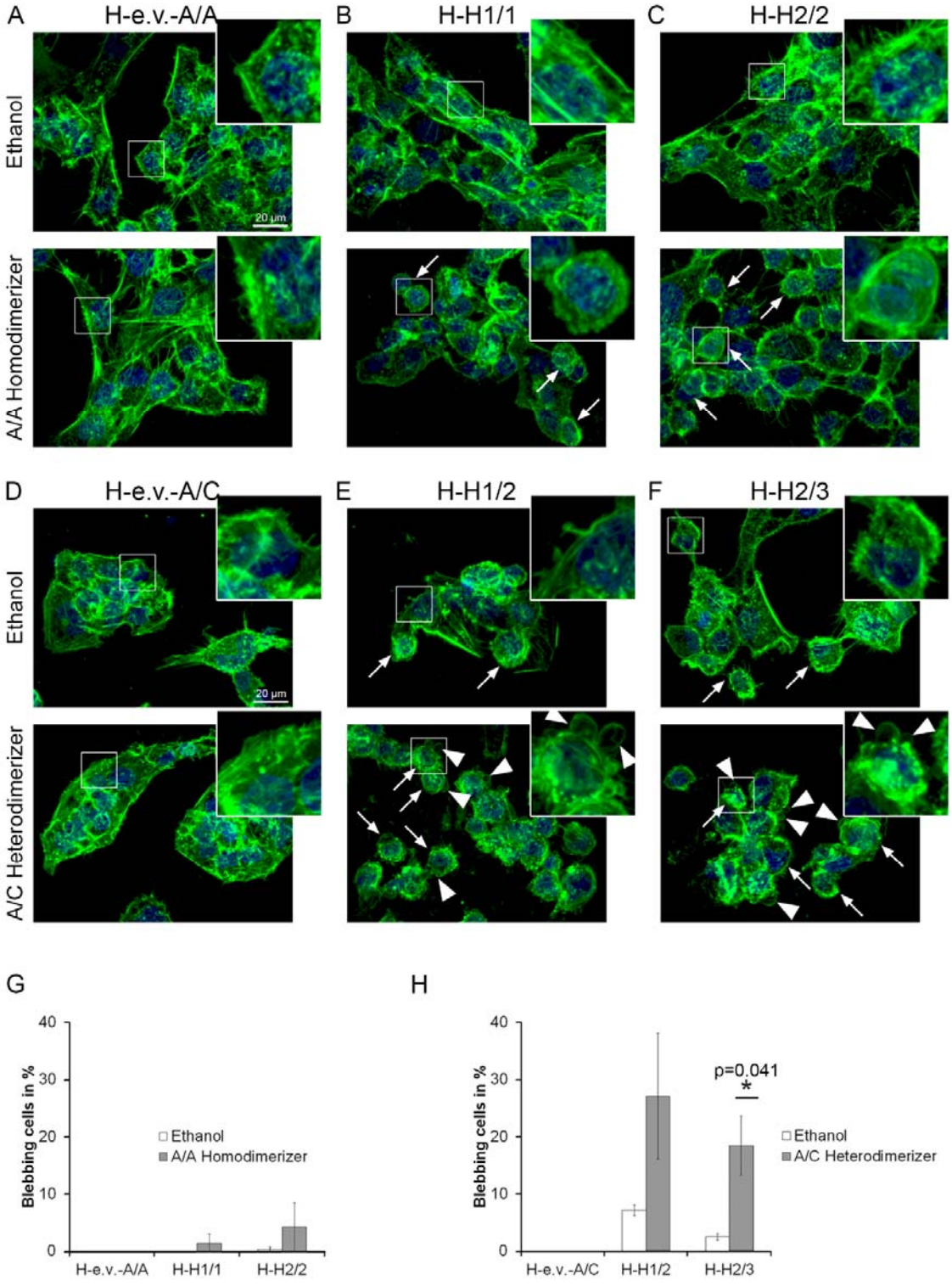


Figure 4: Stimulation of H-H1/1 and H-H2/2 cells in OTCs induced EGFR and HER2 phosphorylation and cell invasion. (A-C) OTCs of H-e.v.-A/A **(A)**, H-H1/1 **(B)** and H-H2/2 **(C)** cells treated with ethanol as control (-) or 300 nM A/A Homodimerizer in ethanol (+). Representative images of HE stained OTCs are shown. Phosphorylation of EGFR and HER2 was detected by immunohistochemistry (brown staining). **(A-C)** All upper panels are at the same magnification, Bar = 100 µm. The lower panels with black frame show a magnified view (5x). H&E and immunohistochemistry stains are representative of three independent biological experiments.

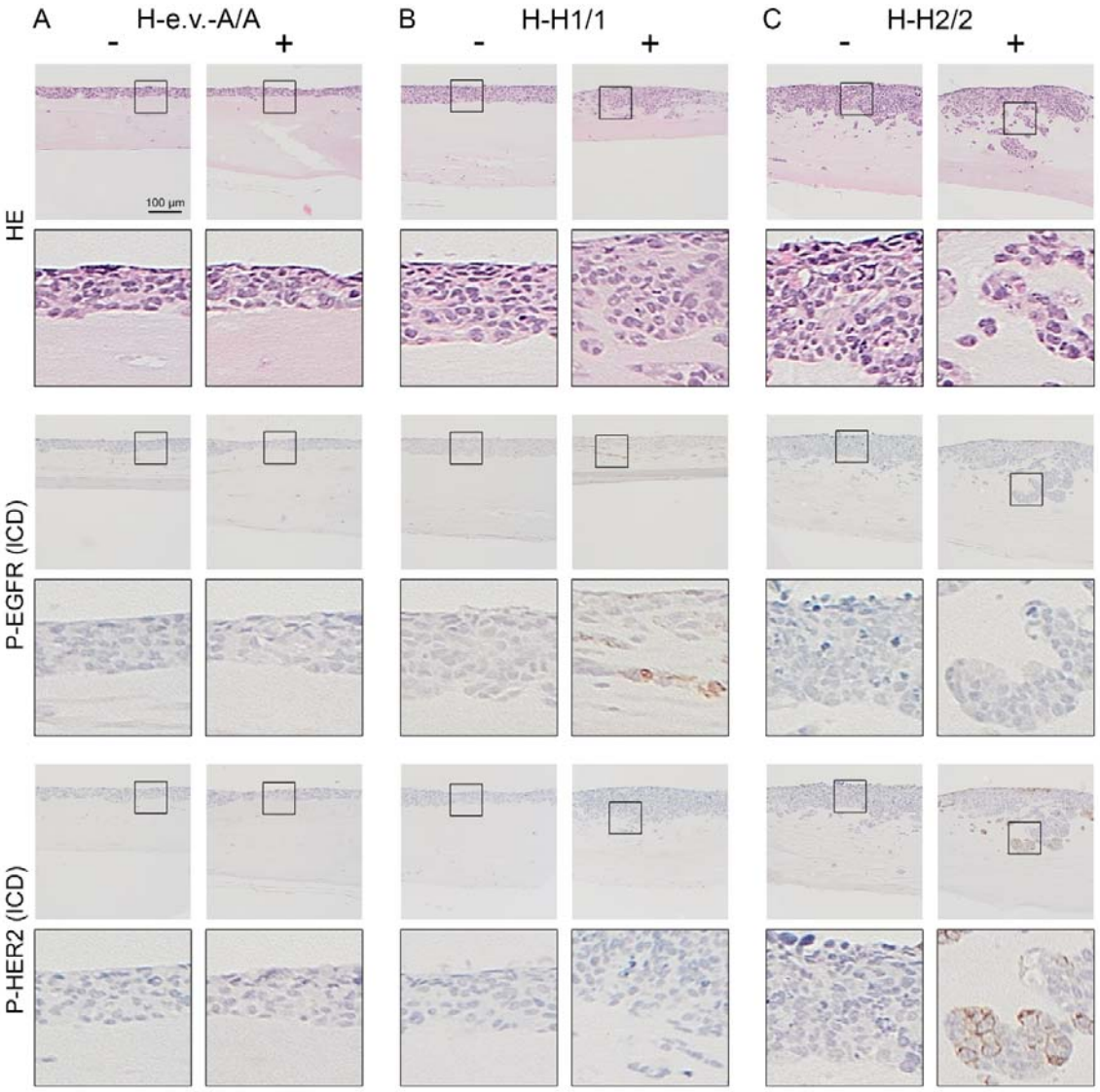


Figure 5: Stimulation of H-H1/2 and H-H2/3 cells in OTCs induced EGFR/HER2 and HER2/HER3 phosphorylation and cell invasion. (A-C) OTCs of H-e.v.-A/C **(A)**, H-H1/2 **(B)** and H-H2/3 **(C)** cells treated with ethanol as control (-) or 300 nM A/C Heterodimerizer in ethanol (+). Representative images of H&E stained OTCs are shown. Phosphorylation of EGFR, HER2 or HER3 was detected by immunohistochemistry (brown staining). **(A-C)** All upper panels are at the same magnification, Bar = 100 μm. The lower panels with black frames show a magnified view (5x). H&E and immunohistochemistry stains are representative of three independent biological experiments.

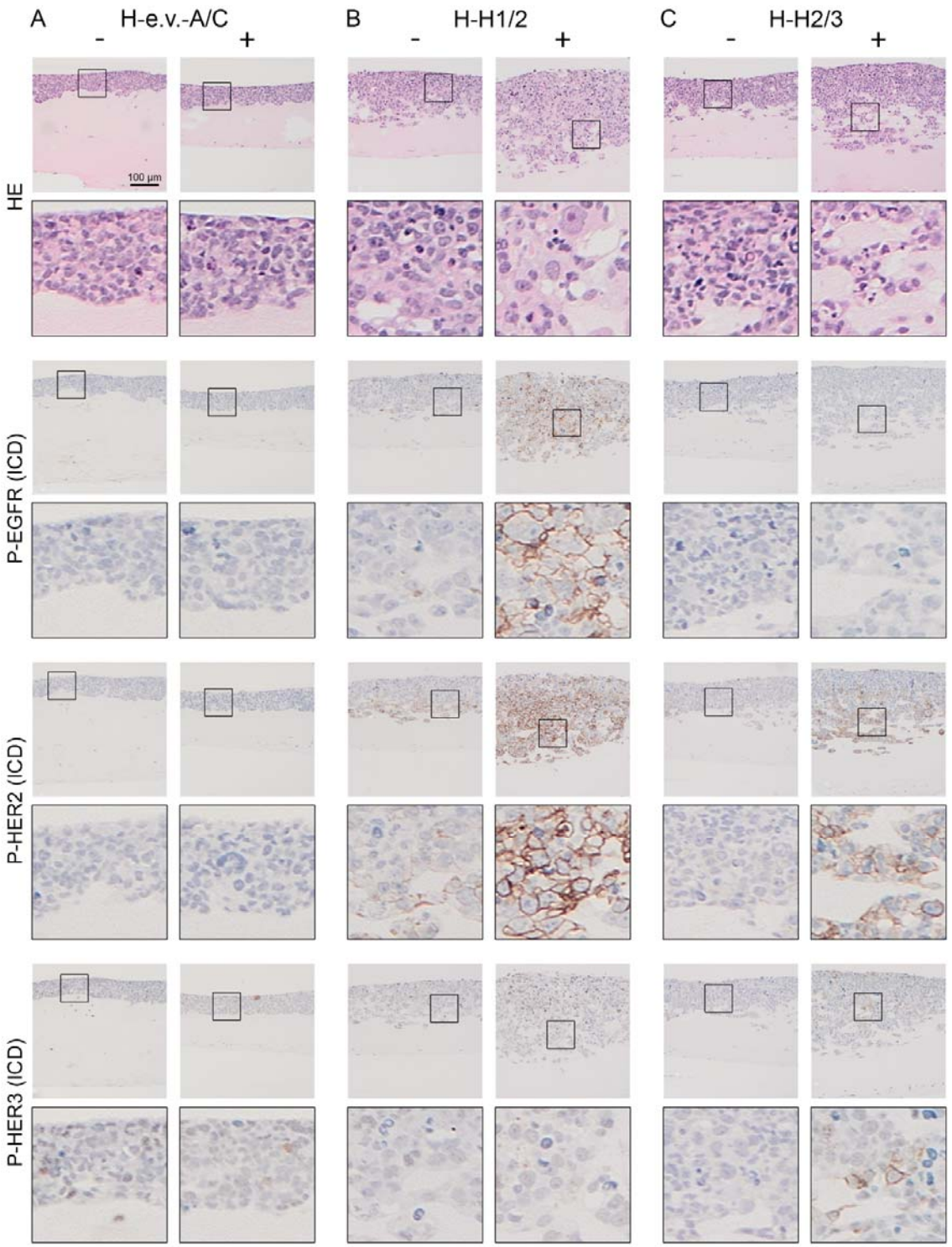
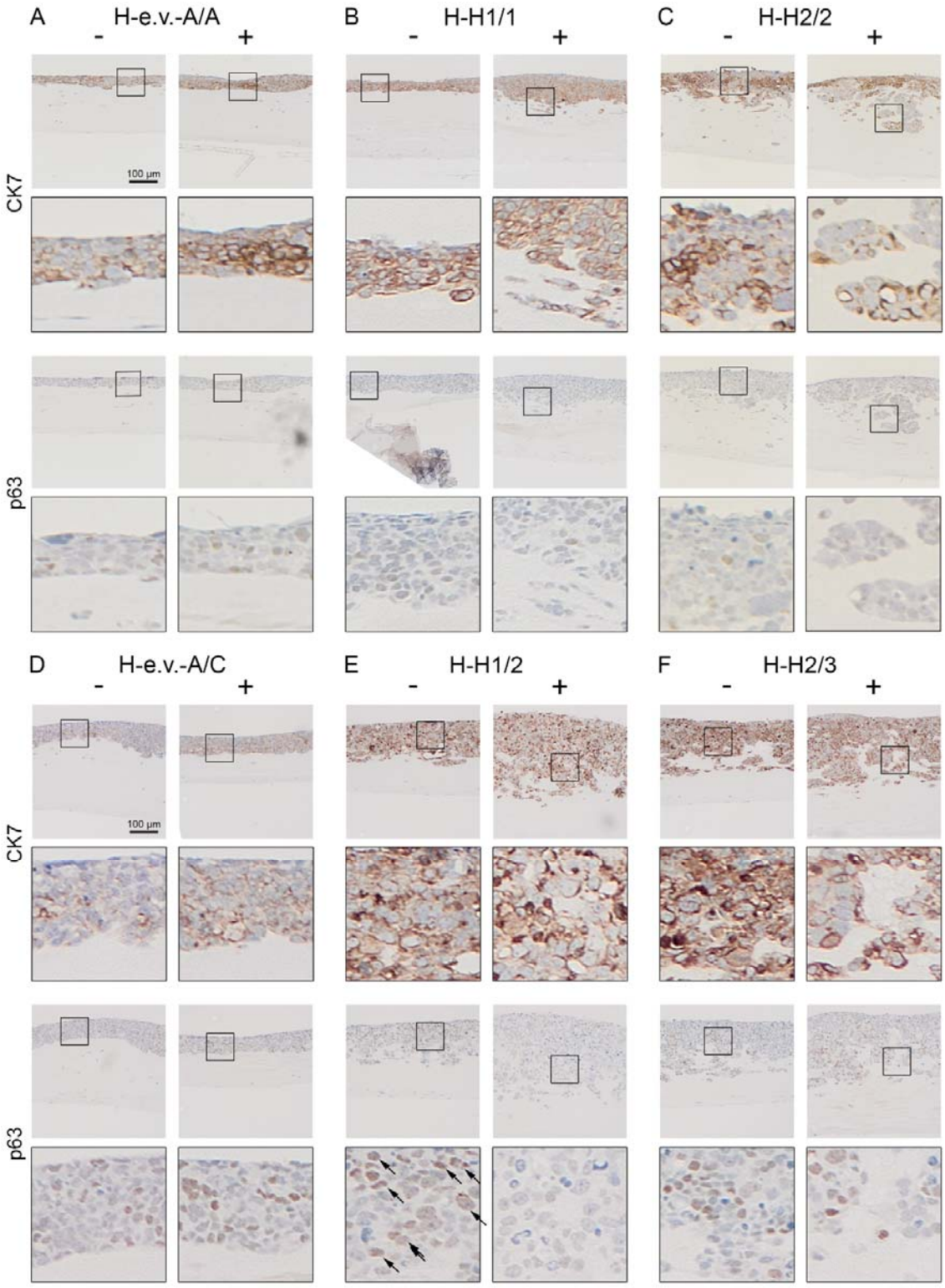


Figure 6: Stimulation of ErbB heterodimers increase CK7 and decrease p63 positivity in H-H1/2 and H-H2/3 cells in OTCs. (A-C) OTCs of H-e.v.-A/A, H-H1/1 and H-H2/2 cells treated with ethanol as control (-) or 300 nM A/A Homodimerizer in ethanol (+). Expression of CK7 (top panel) and p63 (bottom panel) was detected by immunohistochemistry (brown staining). **(D-F)** OTCs of H-e.v.-A/C, H-H1/2 and H-H2/3 cells treated with ethanol as control (-) or 300 nM A/C Heterodimerizer in ethanol (+). Expression of CK7 (top panel) and p63 (bottom panel) was detected by immunohistochemistry (brown staining). **(A-F)** All upper panels are at the same magnification, Bar = 100 μm. The lower panels with black frames show a magnified view (5x). Immunohistochemistry stains are representative of three independent biological experiments.



SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods YES

Supplementary figure legends YES

Figure S1. Membranous expression of fusion proteins in H-e.v.-A/A, H-H1/1, H-H2/2, H-e.v.-A/C, H-H1/2 and H-H2/3 cells and quantification by flow cytometry

Figure S2. A/A Homodimerizer and A/C Heterodimerizer increase fusion protein interaction in H-e.v.-A/A, H-H1/1, H-H2/2, H-e.v.-A/C, H-H1/2 and H-H2/3 cells

Figure S3. H-e.v.-A/A cells are negative for P-EGFR and P-HER2

Figure S4. H-e.v.-A/C cells are negative for P-EGFR P-HER2 and P-HER3

Figure S5. Increased cell proliferation in activated H-H2/2 cells

Figure S6. H-H1/2 and H-H2/3 cells with membrane blebs are not apoptotic and show increased motility in migration and invasion assays

Figure S7. Dimerization induces migration of H-H1/2 and H-H2/3 cells and invasion of H-H2/3 cells

Figure S8. Stimulation of ErbB homo- or heterodimers does not impact Ki-67 positivity but decreases FAK positivity in H-H1/2 and H-H2/3 cells in OTCs

Figure S9. Exploratory MALDI-FT-ICR MSI of organotypic cultures from H-e.v.-A/C and HER2/HER3 cells

Table S1. Primary antibodies used in this study

Table S2. Phosphorylation sites and proteins altered by induction of dimerization