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Mitochondrial HER2 stimulates respiration and promotes tumorigenicity

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Abstract

Background: Amplification of HER2, a receptor tyrosine kinase and a breast cancer-linked oncogene, is associated with aggressive disease. HER2 protein is localised mostly at the cell membrane, but a fraction translocates to mitochondria. Whether and how mitochondrial HER2 contributes to tumorigenicity is currently unknown.

Methods: We enriched the mitochondrial (mt-)HER2 fraction in breast cancer cells using an N-terminal mitochondrial targeting sequence and analysed how this manipulation impacts bioenergetics and tumorigenic properties. The role of the tyrosine kinase activity of mt-HER2 was assessed in wild type, kinase-dead (K753M) and kinase-enhanced (V659E) mtHER2 constructs.

Results: We document that mt-HER2 associates with the oxidative phosphorylation system, stimulates bioenergetics and promotes larger respiratory supercomplexes. mt-HER2 enhances proliferation and invasiveness in vitro and tumour growth and metastatic potential in vivo, in a kinase activity-dependent manner. On the other hand, constitutively active mt-HER2 provokes excessive mitochondria ROS generation, sensitises to cell death, and restricts growth of primary

Eliska Novotna, Mirko Milosevic, and Dana Prukova contributed equally to this work.

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tumours, suggesting that regulation of HER2 activity in mitochondria is required for the maximal pro-tumorigenic effect.

Conclusions: mt-HER2 promotes tumorigenicity by supporting bioenergetics and optimal redox balance.

KEYWORDS

cancer, electron transport chain, HER2, mitochondria, reactive oxygen species

1 | INTRODUCTION

Tyrosine receptor kinase HER2, encoded by the *ERBB2* gene, is a major breast cancer oncogene. The *ERBB2* gene is amplified in more than 20% of breast cancer cases, leading to high expression/activity of the HER2 protein and poor prognosis.[1] HER2 is localised at the plasma membrane and amplifies signals originating from other members of the epidermal growth factor family. Growth-promoting signalling in HER2-driven cancer is autonomous and sustained. HER2 drives cancer growth and survival via MAP kinase and PI3K/AKT pathways, impacting multiple cellular processes including energy metabolism.[2] Interestingly, HER2 and other members of its signalling pathway have also been found in mitochondria.[3]

Mitochondria are organelles involved in ATP production, reactive oxygen species generation (ROS) and catabolic/anabolic metabolism. These functions are linked to oxidative phosphorylation (OXPHOS), a key mitochondrial system that resides at the inner mitochondrial membrane (IMM) and consists of the electron transport chain (ETC) and ATP synthase. The ETC is formed by four respiratory complexes (CI-CIV) that further associate into higher molecular structures called supercomplexes to facilitate respiration,[4,5] and maintains a proton gradient on the IMM that allows ATP synthase (complex V, CV) to produce ATP. The ETC is also a major source of ROS.[6] A fraction of the HER2 protein has been identified at the matrix side of the IMM, where it is targeted by an internal mitochondria-localisation sequence in an mtHSP70-dependent manner.[7,8] This brings HER2 to the proximity of OXPHOS, suggesting that HER2 could modulate OXPHOS via direct interaction. Such modulation of OXPHOS function by mitochondrial HER2 would be of consequence, because HER2 is present in mitochondrial of human breast cancer tumours.[7] At present, the impact of mt-HER2 on mitochondrial function is unclear, with both increased and decreased rate of respiration reported, although in different experimental models.[7,8] Furthermore, it is not known if and how mt-HER2 contributes to the severity of breast cancer.

In this study, we used mitochondria targeted HER2 constructs to selectively deliver HER2 with normal, enhanced, or ablated tyrosine kinase activity into mitochondria of breast cancer cells. Using in vitro and in vivo model systems we aimed to systematically examine how mt-HER2 and its activity affects mitochondrial respiration and how this impacts tumour formation and metastases.

2 | METHODS

2.1 | Cell lines

Human breast adenocarcinoma MCF-7 cell line was obtained from the ATCC. MDA-MB-231 cell line was obtained from J. A. López (Griffith University, Australia). Both cell lines were authenticated using STR analysis, and were cultivated in DMEM (Sigma-Aldrich, D6429) with 10% FBS (Sigma-Aldrich, F7524), 100 U/mL Penicillin (Sigma-Aldrich, P3032) and 100 µg/mL Streptomycin (Sigma-Aldrich, S9137) at 37°C and 5% CO_2. Transfected cell lines (wt-HER2, mt-HER2, mt-HER2^{K753M} and mt-HER2^{V659E}) were cultivated in media with puromycin (.12 µg/mL, InvivoGen, ant-pr). Medium without puromycin was used for experiments to provide the same conditions across cell lines. Cells were passaged every 2–3 days using trypsin solution (.25% trypsin, .01% EDTA).

2.2 | DNA constructs

Construction of wild type HER2 and mitochondria targeted HER2 plasmids containing N-terminal COX8 mitochondrial targeting sequence was described before.[8] Kinase mutants of mt-HER2 (mt-HER2^{K753M} and mt-HER2^{V659E}) were generated in the mts-HER2 pEF/IRES/Puro plasmid by quick change mutagenesis, using the following primers (mutated sites underlined): 5′-gac cag cag aat gcc ctc cac cgc aga gac ga-3′ (mt-HER2^{V659E} F), 5′-tcg tct ctg cgg tgg agg gca ttc tgc tgg tc-3′ (mt-HER2^{V659E} R), 5′-gat gtg ttt tcc ctc aac acc atg atg gcc act gga att ttc-3′ (mt-HER2^{K753M} F), 5′-gaa aat tcc agt ggc cat cat ggt gtt gag gga aaa cac atc-3′ (mt-HER2^{K753M} R) and transformed into TOP10 *E. coli* (C404003). The pLenti6.3/V5 lentiviral plasmid containing mitochondria-targeted catalase and

blasticidin resistance was a kind gift of S Lortz, (Hannover Medical School).[9] cdDNA for human SOD2 was a kind gift of F. Domann (University of Iowa). The SOD2 ORF was PCR amplified using primers ATA AAG CTA GCc acc ATG TTG AGC CGG GCA GTG TG (F) and ATA AAG AAT TCT TAC TTT TTG CAA GCC ATG TAT C (R) and cloned into the pCDH-MCS-Puro lentiviral plasmid using NheI/EcoRI restriction sites. Lentiviral particles containing mt-CAT and SOD2 constructs were produced and used as described.[10] Luciferase constructs and lentiviruses were described previously.[11]

2.3 | Cell line transfection

Cell lines were seeded in a six well plate. A day after seeding the cells were transfected with 2 μg of plasmid DNA using Lipofectamin 3000 Transfection Reagent (Invitrogen, L3000008) in Opti-MEM medium (Gibco, 31985070). 24 h later the cells were plated onto 150 mm dishes and selected with puromycin (.25 μg/mL). Individual colonies were collected and tested for HER2 expression by western blotting (WB).

2.4 | Tumour growth and metastasis formation

The MDA-MB231 sublines transduced with luciferase were injected into the mammary fat pads on both sides of 7-week-old female NSG-SGM3 mice (5×10^5 cells per side in PBS/Matrigel, Corning 356230). Tumour size was measured by callipers twice a week, combining both tumours from each animal to obtain one size value per animal. The animals maintained constant body weight throughout the experiment. Mice were sacrificed on day 28. To assess metastatic activity, the animals were injected intraperitoneally with luciferin (PerkinElmer, 122799) at 15 mg/mL (10 μL/g mouse body weight), anaesthetised by isoflurane inhalation (Aerrane, Baxter) and sacrificed after 5 min. The lungs were excised and visualised ex-vivo using bioluminescence (LagoX, Spectral Instruments Imaging). After that, lungs were fixed, embedded in paraffin, sectioned, and stained with haematoxylin/eosin. Animals were maintained under SPF conditions in individually ventilated cages with controlled temperature (22 ± 2°C) and humidity under a 12 h light/12 h dark cycle and with food and drink ad libitum. Animals were closely followed-up by the animal caretakers and the experimenters, with regular inspection by a veterinarian, as per the standard health and animal welfare procedures of the local animal facility. No statistical method was used to pre- determine sample size. All animal experiments were approved by the Animal Ethics Committee of the Czech Academy of Sciences and were performed according to Czech guidelines for the Care and Use of Animals in Research and Teaching. None of the animals showed signs of distress during the experiments.

2.5 | Statistical analysis

Data were analysed in GraphPad Prism 9 software (GraphPad Software) using unpaired Student's *t*-test analysis for pair comparisons, and one-way ANOVA or two-way ANOVA for comparisons of more than two parameters. Data shown are mean values ± SEM of at least 3 independent experiments. A statistical difference of $p < .05$ was considered significant.

2.6 | Other methods

SDS-PAGE and western blotting, native blue gel electrophoresis, complexome analysis, immunoprecipitation, and mass spectrometry, high-resolution respirometry, glucose uptake and lactate production, measurements of ROS production and mitochondria membrane potential, cell death assessment, cell proliferation, scratch-wound healing assay and immunogold transmission electron microscopy are described in the Appendix S1.

3 | RESULTS

3.1 | Mitochondrial HER2 stimulates metabolism and bioenergetics in breast cancer cells

To address the role of mitochondrial HER2 in mitochondrial function, we generated breast cancer cell lines stably expressing wild type HER2 (wt-HER2), or HER2 targeted into mitochondria (mt-HER2) by fusing the mitochondria-targeting sequence of COX8 to the N terminus of HER2.[8] As a primary cellular model, we selected the MDA-MB-231 triple-negative breast cancer cell line, which has little or no endogenous HER2 expression (Figure 1A). In addition, for some experiments we also used oestrogen-dependent MCF-7 cell line to rule out cell line-specific effects (Figure S1A). Transmission electron microscopy revealed increased immunogold labelling of HER2 in the cytoplasm and at the plasma membrane of wt-HER2 and mt-HER2 expressing MDA-MB-231 cells. In contrast, only mt-HER2 expression significantly increased the HER2 signal in the mitochondria (Figure 1B–D and Figure S2A,B), pointing to a relative enrichment of HER2

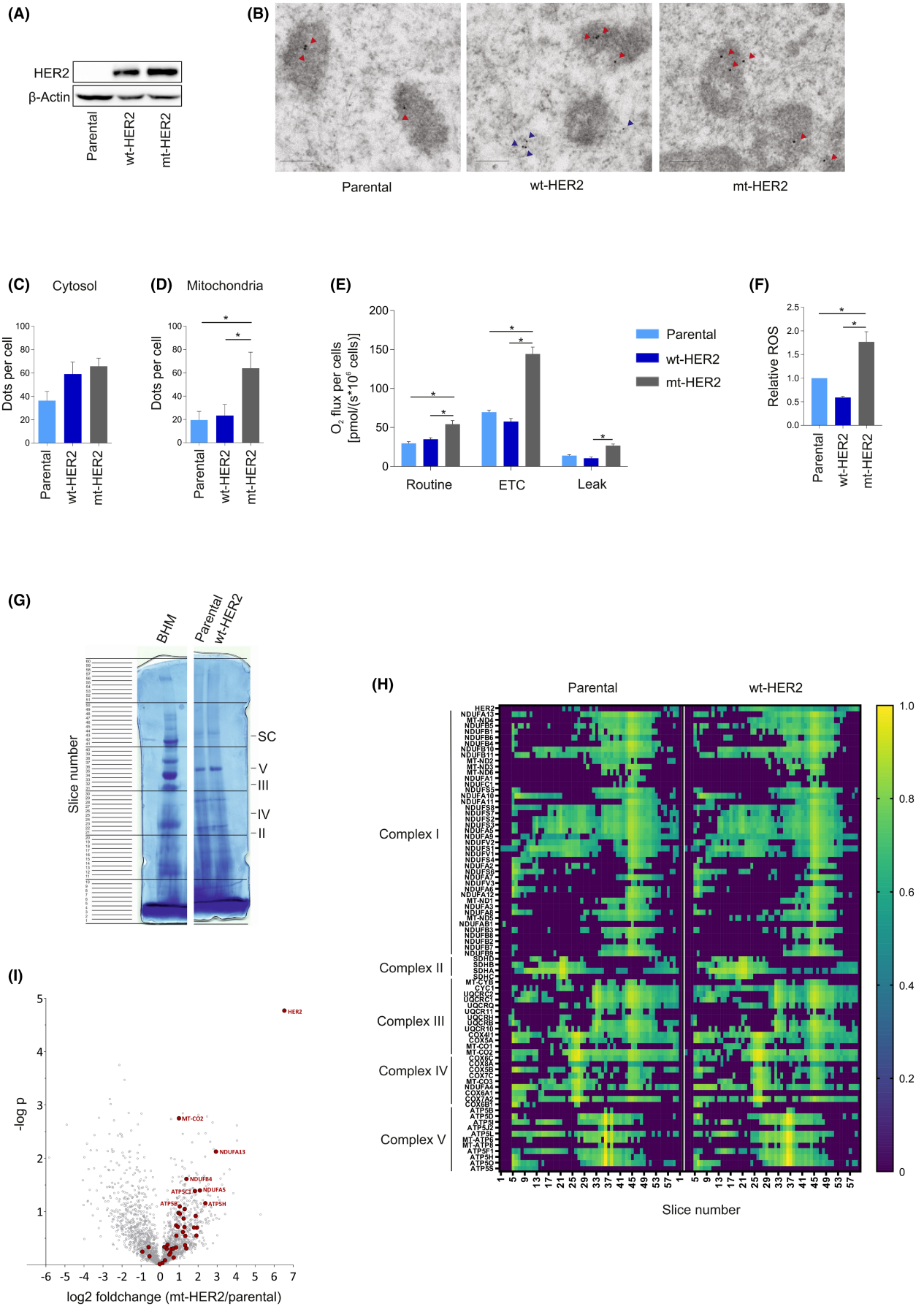


FIGURE 1 Mitochondrial HER2 stimulates bioenergetics in MDA-MB-231 breast cancer cells. (A) Representative WB images ($n = 3$) of HER2 protein expression in MDA-MB-231 parental cells and in sublines expressing HER2 variants with wild type and mitochondria targeted expression. β-Actin was used as a loading control. (B–D) Representative transmission electron microscopy images with immunogold staining of HER2 in MDA-MB-231 sublines (B) and quantification of HER2 in cytosol (C) and mitochondria (D). Red and blue arrows mark HER2 in mitochondria and cytosol, respectively (mean ± SEM, $n = 6$ cells per subline, multiple sections were analysed for each labelling experiment, $^*p < .05$, one-way ANOVA with Sidak's multiple comparison test). Scale bar, .2 μm. (E) Routine, ETC and leak respiration of MDA-MB-231 sublines measured by oxygraph (mean ± SEM, $n \geq 5$, $^*p < .05$, two-way ANOVA with Tukey's multiple comparisons test). (F) ROS levels in MDA-MB-231 sublines assessed using DCF-DA fluorescent probe by flow cytometry (mean ± SEM, $n = 6$, $^*p < .05$, one-way ANOVA with Tukey's multiple comparisons test). (G) Native blue gel electrophoresis (NBGE) of mitochondria in the indicated MDA-MB-231 sublines. The position of respiratory complexes II–V and supercomplexes (SC) of bovine heart mitochondria (BHM) is shown. The gel was cut as indicated on the left and the protein content of individual slices was analysed by LC–MS. (H) Relative protein expression in the native gel shown in (G) measured by LC–MS. *x*-axis indicates the position on the native gel. Results are from 1 lane of NBGE. (I) HER2 interacting partners immunoprecipitated with an anti-HER2 antibody in MDA-MB-231 sublines. HER2 and subunits of OXPHOS are shown by red dots ($n = 3$ independent immunoprecipitations).

in the mitochondrial compartment of mt-HER2 compared to wt-HER2 cells.

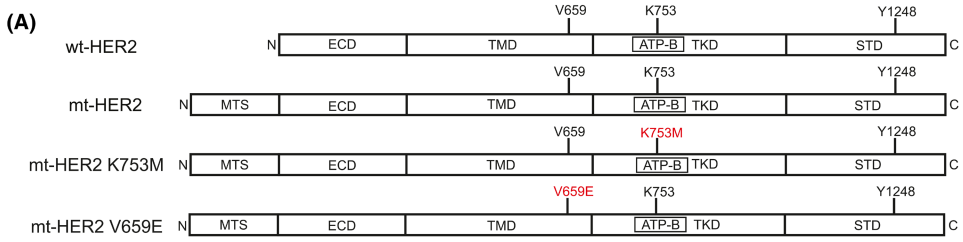
Next, we examined cellular bioenergetics. Compared to corresponding parental cells or to cells expressing wt-HER2, mt-HER2 expressing cells showed increased oxygen consumption (Figure 1E and Figure S1B) and elevated ROS levels (Figure 1F and Figure S1C). This documents a stimulating effect of mt-HER2 on mitochondrial respiration independent of the cellular background.

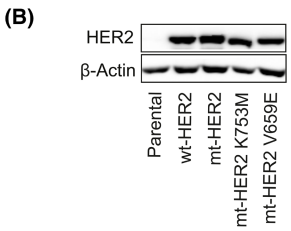
3.2 | Mitochondrial HER2 interacts with components of oxidative phosphorylation

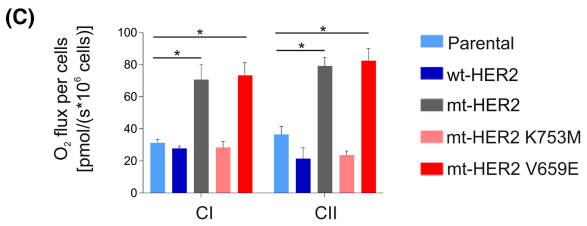
Mt-HER2 stimulates respiration as well as ROS production and is localized at the IMM,[7,8] suggesting that it could directly interact with OXPHOS. To assess possible mt-HER2 interaction with OXPHOS in a native state, we performed complexome analysis. Proteins in isolated mitochondria were extracted by digitonin, a mild detergent that preserves native protein complexes, and resolved using native blue gel electrophoresis (NBGE). After separation, individual lanes of the NBGE gel were sectioned into small segments and distribution of proteins in the individual segments was analysed by mass spectrometry. Proteins that form a complex co-migrate together and are therefore detected in the same segment.[12] Analysis of the mitochondrial proteins isolated from MDA-MB-231 cells revealed that overexpressed HER2 co-migrated with two subcomplexes of CI (Figure 1G,H), possibly assembly intermediates. Furthermore, HER2 co-migrated with assembled CIV and with ATP synthase. Similar findings were obtained when we analysed mitochondria of MCF7 cells (Figure S1D,E). As colocalisation on blue native gels suggests an interaction but does not directly prove it, we next performed immunoprecipitation using IgG against HER2 in mt-HER2-expressing MDA-MB-231 cells. Mass spectrometry analysis showed that the immunoprecipitate was enriched for multiple subunits of respiratory complexes, most notably those of CI (NDUFA13, NDUFB4, NDUFA5), CIV (MT-COX2) and ATP synthase (ATP5H, ATPC1, ATP5B) (Figure 1I and Table S1). These data suggest that mt-HER2 interacts with several OXPHOS complexes.

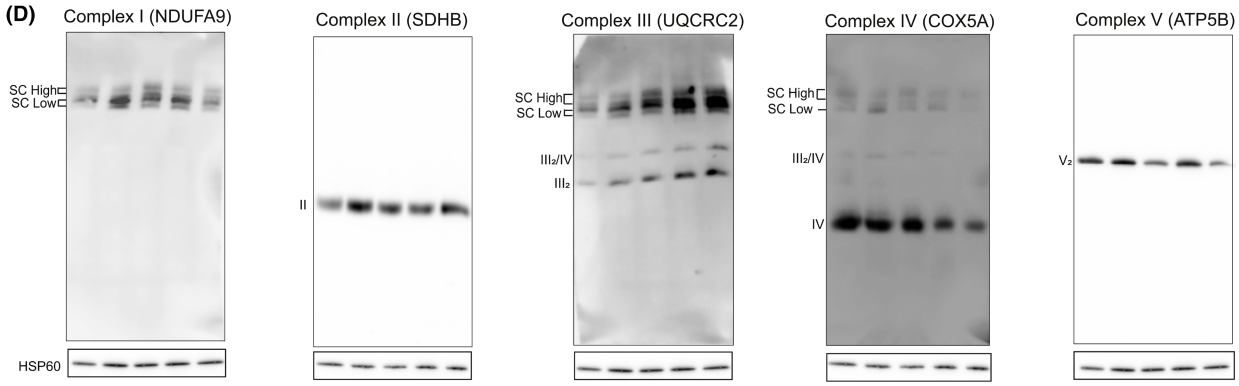
3.3 | Kinase activity of mt-HER2 stimulates bioenergetics

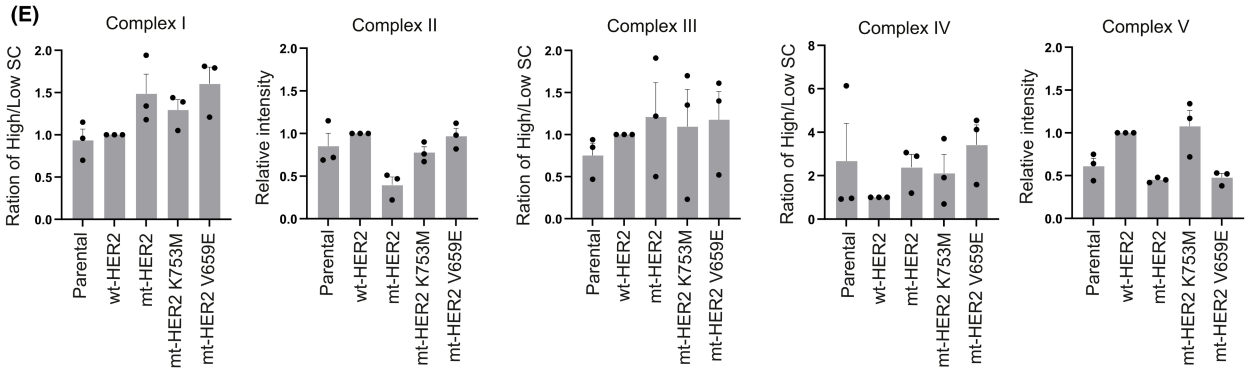
We next investigated if the kinase activity of mt-HER2 is required for the stimulation of respiration. We stably transfected MDA-MB-231 and MCF7 cells with mt-HER2 variants containing point mutations that either abrogate (K753M) or enhance (V659E) its tyrosine kinase activity (Figure 2A,B).[7] On the MCF7 background we could not obtain viable V659E clones (see Section 4), and we thus primarily focused on the MDA-MB-231 system. Interestingly, whereas the kinase-enhanced mt-HER2^{V659E} variant stimulated oxygen consumption similarly to unmodified mt-HER2, the kinase-dead mt-HER2^{K753M} had no effect (Figure 2C). Next, we assessed whether the presence of mt-HER2 modulates the assembly of respiratory complexes of the OXPHOS system into supercomplexes that were previously proposed to optimise respiration and substrate utilisation.[4,13] Consistent with increased oxygen consumption in mt-HER2 and mt-HER2^{V659E} cells, distribution of their respiratory supercomplexes was slightly shifted towards higher molecular weight species compared to parental, wt-HER2 or mt-HER2^{K753M} cells. When expressed relatively as a ratio of higher (larger) and lower (smaller) supercomplexes, this shift was most apparent for supercomplexes containing CI. In contrast, the level of assembled ATP synthase (CV), which is not a part of the ETC, was reduced. (Figure 2D,E). In addition, the mitochondrial membrane potential was elevated in mt-HER2 and mt-HER2^{V659E} cells (Figure 2F). Interestingly, mt-HER2 not only stimulated OXPHOS, but also glycolysis. Mt-HER2^{V659E} cells, and to a lesser degree also mt-HER2 cells, took up more glucose (Figure 2G) and secreted more

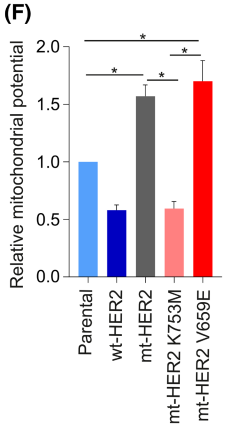


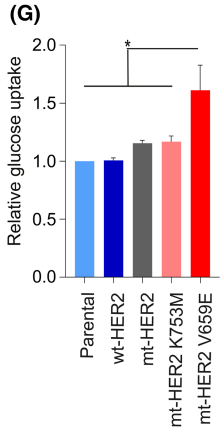












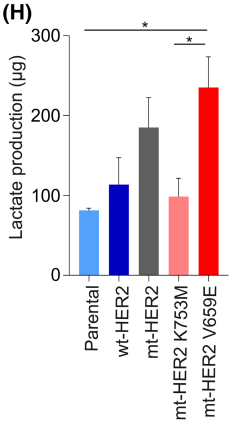


FIGURE 2 Stimulation of bioenergetics by mt-HER2 in MDA-MB-231 cells depends on its kinase activity. (A) A structure of HER2 constructs used in the study. Point mutations were introduced in mt-HER2 to produce HER2 kinase-dead (mt-HER2^{K753M}) and kinase-enhanced (mt-HER2^{V659E}) variants. ATP-B, ATP-binding site; ECD, extracellular domain; MTS, mitochondria targeting sequence; STD, signal transducing domain; TKD, tyrosine kinase domain; TMD, transmembrane domain. Mutated residues are shown in red. (B) Representative WB image ($n = 3$) of HER2 protein levels in MDA-MB-231 sublines. β-Actin was used as a loading control. (C) Respiration via complex I and II in MDA-MB-231 sublines (mean ± SEM, $n \geq 3$, $^*p < .05$, two-way ANOVA with Tukey's multiple comparisons test). (D) Representative WB images of OXPHOS complexes/supercomplexes (SC) resolved by NBGE and detected by IgG against indicated subunits. HSP60 was used as a loading control ($n = 3$ independent experiments). (E) Quantification of the NBGE in (D) relative to wt-HER2. Data for complexes I, III and V are presented as a ratio of higher to lower SC (mean ± SEM, $n = 3$). (F) Mitochondrial membrane potential in MDA-MB-231 sublines shown as a ratio of tetramethylrhodamide (TMRM) and MitoTracker Deep Red (MDR) signal and measured by flow cytometry (mean ± SEM, $n = 5$, $^*p < .05$, one-way ANOVA with Tukey's multiple comparisons test). (G) Relative glucose uptake in MDA-MB-231 cells (mean ± SEM, $n = 5$, $^*p < .05$, one-way ANOVA with Tukey's multiple comparisons test). (H) Lactate production in MDA-MB-231 cells (mean ± SEM, $n \geq 5$, $^*p < .05$, one-way ANOVA with Tukey's multiple comparisons test).

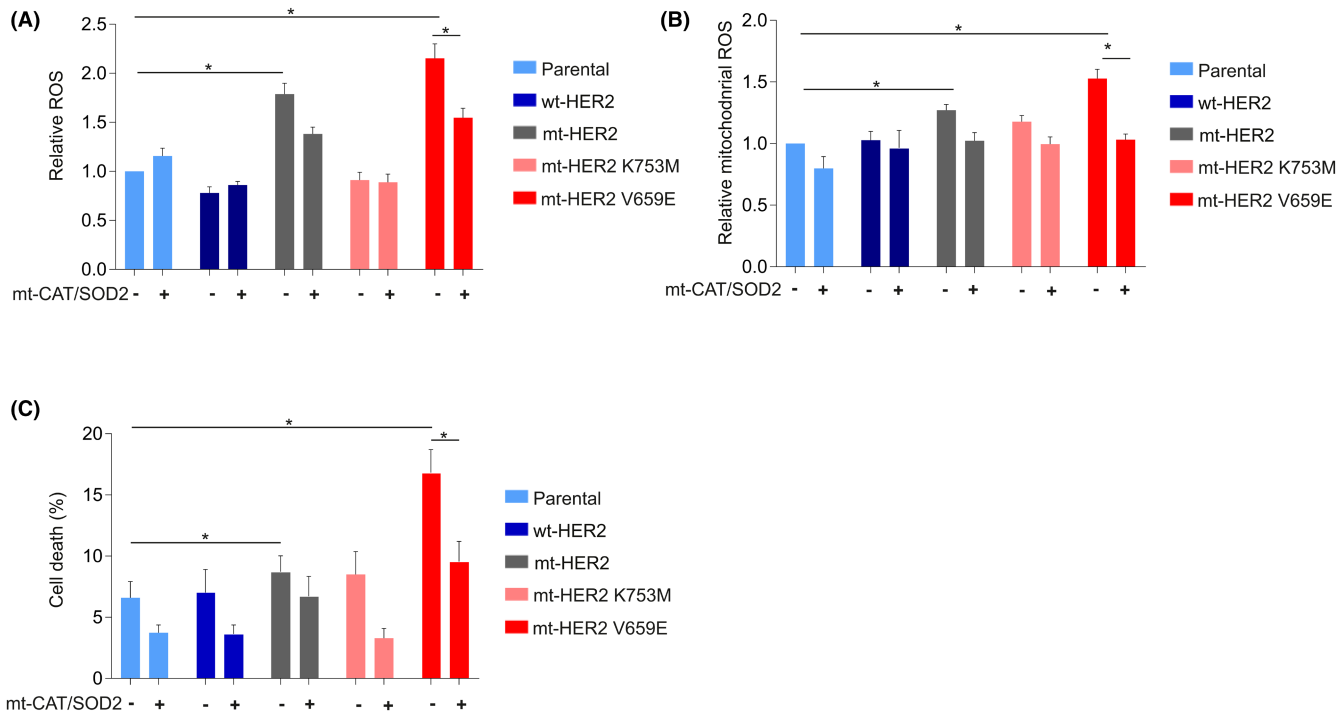


FIGURE 3 Kinase-enhanced mt-HER2 variant stimulates excessive ROS production and cell death. (A,B) ROS levels in indicated MDA-MB-231 sublines with or without overexpression of mitochondria targeted catalase and superoxide dismutase 2 (mt-CAT/SOD2) assessed by DCF-DA (A) and MitoSOX (B). (C) Cell death in MDA-MB-231 cells overexpressing or not mt-CAT/SOD2 evaluated by Annexin V/Hoechst using flow cytometry. In (A–C) data represent mean ± SEM, $n \geq 6$, $^*p < .05$, one-way ANOVA with Dunnett's multiple comparison test, or unpaired two-tailed *t*-test for pairwise comparisons.

lactate (Figure 2H) than parental cells, suggesting that mt-HER2 also impacts cellular energy metabolism. In summary, tyrosine kinase activity of mt-HER2 stimulates bioenergetics and activates metabolism.

3.4 | Kinase-enhanced mt-HER2 variant stimulates excessive ROS production

As the ETC is a major source of ROS, we next assessed if mt-HER2 variants differentially modulate generation of ROS from the ETC. Compared to parental cells, intracellular ROS levels were increased in mt-HER2^{V659E} cells and in cells expressing mt-HER2, albeit to a lesser degree (Figure 3A). Consistent with the effect of mt-HER2 on ROS generation by the ETC, MitoSOX, a mitochondrial ROS probe, revealed an increase of mitochondrial ROS signal in mt-HER2^{V659E} cells. Mt-HER2 also elevated mitochondrial ROS but less prominently, whereas the other HER2 variants had no effect (Figure 3B). Since high intracellular ROS levels are toxic, we investigated if elevated ROS in kinase-enhanced mt-HER2^{V659E} expressing cells could compromise their viability. Indeed, we observed increased rate of constitutive cell death in

presence of mt-HER2^{V659E} (Figure 3C), while such effect was not observed for the other mt-HER2 variants. Similarly, mt-HER2^{V659E} cells were sensitised to an exogenous ROS inducer MitoTam,[8,14] an anti-cancer agent currently in clinical trials (Figure S3A). To address whether increased mitochondrial ROS in mt-HER2^{V659E} cells are responsible for the elevated rate of cell death, we stably expressed superoxide dismutase (SOD2) together with mitochondria targeted catalase (mt-CAT) (Figure S3B). This combination reduced ROS levels both in the mitochondria and in cytoplasm of mt-HER2^{V659E} cells but not in cells expressing the other HER2 variants (Figure 3A,B). These data confirm that increased ROS levels in mt-HER2^{V659E} cells are of mitochondrial origin. Importantly, SOD2/mt-CAT also decrease the level of spontaneous cell death in mt-HER2^{V659E} cells (Figure 3C), suggesting that constitutive mt-HER2 activity stimulates excessive mitochondrial ROS generation that predisposes to cell death.

3.5 | mt-HER2 confers pro-tumorigenic properties

Having established that mt-HER2 stimulates bioenergetics, metabolism as well as ROS generation in a tyrosine kinase-dependent manner, we investigated whether mt-HER2 confers pro-tumorigenic properties. In MDA-MB-231 cells mt-HER2 and kinase enhanced mt-HER2^{V659E} significantly stimulated both proliferation (Figure 4A) and migration (Figure 4B) in 2D cultures. In contrast, the kinase-dead mt-HER2^{K753M} or wt-HER2 had little or no effect. Consistently, mt-HER2, but not kinase-dead mt-HER2^{K753M}, stimulated proliferation and migration (Figure S4A,B) in MCF7 cells (kinase-enhanced mt-HER2^{V659E} is not available on the MCF7 background). Hence, we conclude that the kinase-activity of mt-HER2 promotes processes associated with tumorigenicity.

3.6 | mt-HER2 promotes tumour growth and metastasis

Next, we examined the effect of mt-HER2 expression on primary tumours and their metastatic dissemination by grafting luciferase-transduced MDA-MB-231 cells into the mammary fat pad of immunodeficient mice. Compared to parental and wt-HER2 cells, the mt-HER2-expressing cells produced tumours that grew faster and became larger (Figure 5A), whereas mt-HER2^{K753M} had no effect. In contrast to in vitro results, mt-HER2^{V659E} did not provide any growth advantage in vivo. To examine the metastatic potential, we focused on lungs, a major metastatic site for MDA-MB-231 cells. Lungs from the mice above were excised to overcome the low sensitivity of whole-body imaging, and metastases were analysed ex vivo by bioluminescence and histochemistry. Unlike in most other experiments where parental and wt-HER2 cells behaved similarly, the presence of wt-HER2 reduced the metastatic potential of MDA-MB-231 cells (Figure 5B). Accordingly, to assess the

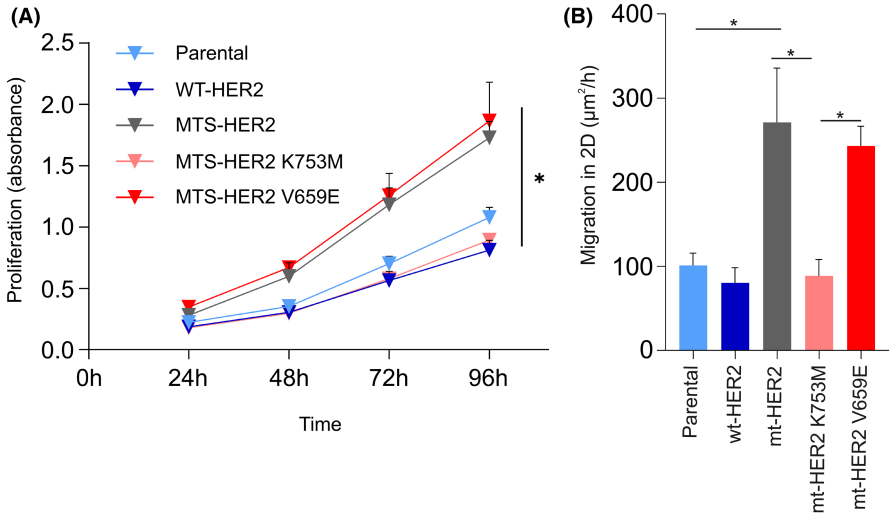


FIGURE 4 Mt-HER2 promotes tumorigenicity. (A) Proliferation of MDA-MB-231 sublines evaluated using crystal violet staining (first measurement at 24 h, mean ± SEM, $n = 4$, *$p < .05$ MDA-MB-231 parental compared to mt-HER2 and mt-HER2^{V659E}, two-way ANOVA with Tukey's multiple comparisons test). (B) Migration of MDA-MB-231 sublines in the scratch-wound healing assay expressed as migration speed (mean ± SEM, $n = 4$ independent experiments, 4 fields of view per experiment, *$p < .05$, one-way ANOVA with Tukey's multiple comparisons test).

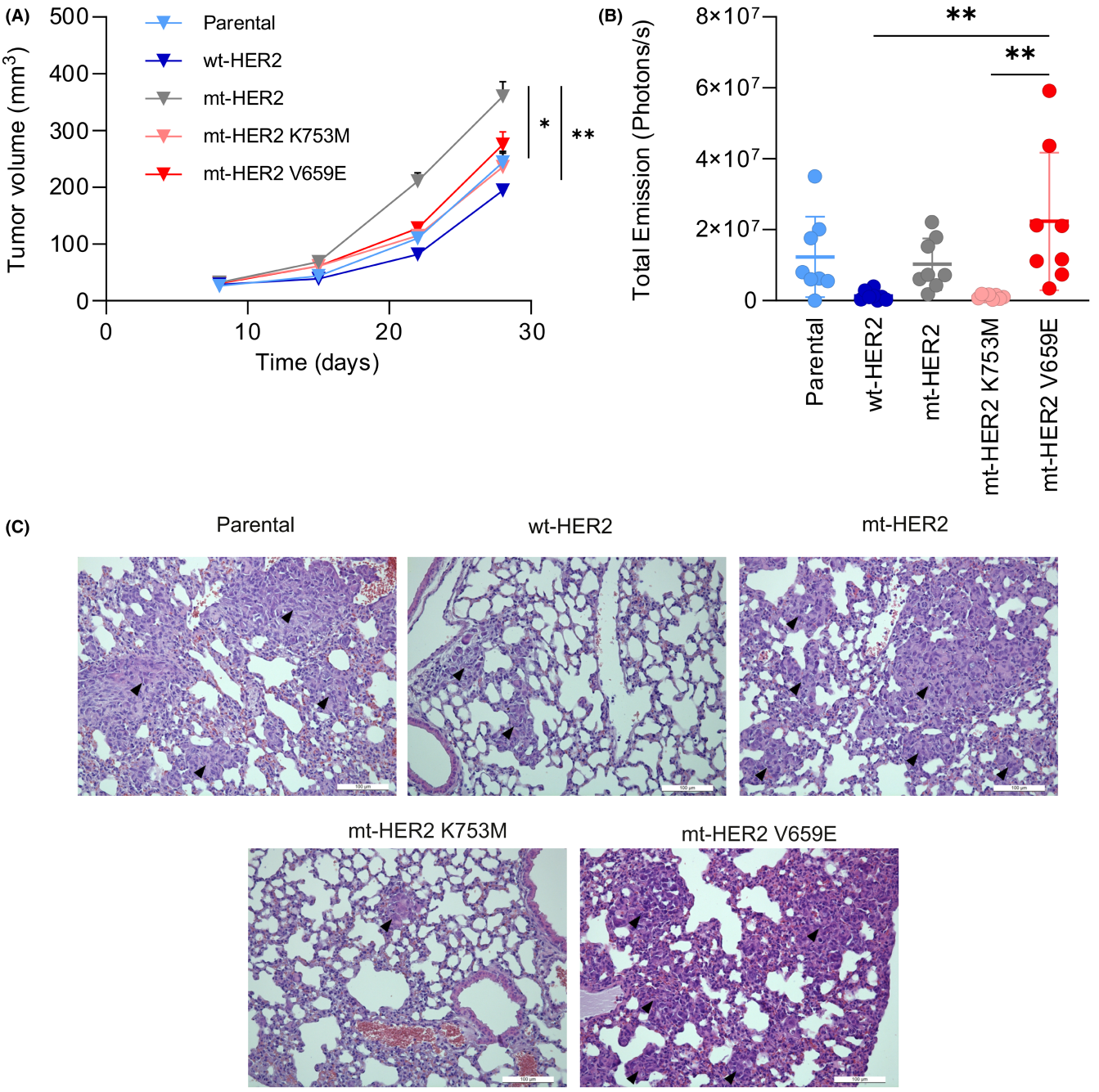


FIGURE 5 Mt-HER2 promotes tumour growth and metastasis. (A) Orthotopic tumours in mammary fat pad generated by MDA-MB-231 sublines grafted into NSG-SGM3 mice (mean ± SEM, $n = 8$ mice, $^*p < .05$ between mt-HER2 and all the other variants, two-way ANOVA with Tukey's multiple comparisons test). (B) Metastatic burden measured in dissected lungs of the mice above using bioluminescence imaging (mean ± SEM, $n = 8$ mice, $^*p < .01$, two-way ANOVA with Tukey's multiple comparisons test). (C) Representative images of metastases in the lungs of mice bearing indicated tumours visualized by haematoxylin eosin staining. Metastasis are marked by black arrowheads.

specific impact of mitochondrial targeting of HER2 on metastasis formation, we used wt-HER2 cells instead of the parental cells (which are HER2-negative, c.f. Figure 2B) as controls for mt-HER2. Compared to wt-HER2, the metastatic burden in the lungs was increased by mt-HER2^{V659E} and by mt-HER2 (although less prominently), but not by mt-HER2^{K753M}, as measured by the total luminescence of isolated lungs (Figure 5B) and by assessing the number of metastases on representative histological sections (Figure 5C). This indicates that mt-HER2 promotes tumour growth and invasiveness in vivo in a kinase activity-dependent manner, yet its activity must be regulated for the maximal pro-tumorigenic effect.

4 | DISCUSSION

While the function and oncogenic activity of HER2 at plasma membrane and the associated signalling pathways are well described and successfully targeted in the clinic, the function of HER2 in mitochondria is less clear. Previously published findings concerning the effect of mt-HER2 on mitochondrial respiration are contradictory[7,8] and the potential role of mt-HER2 in tumorigenicity has not been established. As mt-HER2 is found in human tumours, these aspects are relevant for future treatment strategies targeted at HER2.

The key findings of this study are that mt-HER2 (i) stimulates respiratory function in a kinase activity-dependent manner and (ii) promotes tumorigenicity of breast cancer cells more strongly than wt-Her2. Mt-HER2 may exert these two functions via direct interaction with components of the OXPHOS system, particularly with CI, CIV, and ATP synthase. In CI, HER2 likely binds to its assembly intermediates. CI assembles in a step-wise manner from smaller multi-subunit modules, including the Q, P, Qp and N modules, that form assembly intermediates of increasing size and complexity.[15] The N module is attached last and installs the active site for NADH conversion which enables CI activity. Analysis of immunoprecipitated CI subunits and their co-migration with HER2 in the complexome analysis suggests that mt-HER2 could attach to the nascent Q module first and remain bound in the larger CI sub-assemblies such as Qp until dissociating prior to the N module attachment. mt-HER2 likely binds neither mature CI, nor CI within the supercomplexes. Nevertheless, during the assembly process of CI mt-HER2 could modify the region at the interface of the peripheral and trans-membrane arms of CI (formed by the Q and Qp modules) that is close to the site of ubiquinone reduction.[16] Modulation of this region has been shown to increase the amount of ROS generated from CI.[17] Unlike CI, mt-HER2 may bind to CIV when it is fully assembled, but prior to its insertion into supercomplexes, consistent with the notion that mt-HER2 contributes to more efficient organisation of ETC complexes/supercomplexes during the assembly process. Finally, the mt-HER2 interaction with ATP synthase likely occurs with the fully assembled species.

Our findings thus indicate that mt-HER2 interacts with OXPHOS in a complex manner via CI, CIV and ATP synthase, and not only via CIV as previously suggested.[7] Such complex interaction could underly the contradictory findings regarding the effect of mt-Her2 on bioenergetics. We observed a robust stimulation of respiration by mt-HER2 both in MDA-MB-231 and MCF7 cells, whereas another publication[7] reported downregulation of mitochondrial respiration in the presence of mt-HER2 in MDA-MB-231 cells. Our findings are thus consistent with our previous results[8] that show increased respiration in wt-HER2 MCF7 cells.

Finally, we report here that mt-HER2 is more pro-tumorigenic compared to wt-HER2, which had little effect in our models. The reason for the limited impact of wt-Her2 is unclear at present but might be linked to out of context HER2 signalling or to negative feedback loops.[18,19] The pro-tumorigenic effect of mt-HER2 is consistent with elevated metabolism, stimulation of respiration and moderately increased mitochondrial ROS production, induced in a kinase activity-dependent manner (the kinase-dead variant mt-HER2^{K753M} has no effect). Moderate ROS production by the ETC is essential for proliferation and tumour growth as well as stress resistance,[20–23] and increased OXPHOS in breast cancer favours tumour progression, invasion and metastasis.[24–26] However, excessive ROS levels can interfere with tumorigenicity in vivo and induce cell death.[2,21,27] Compared to unmodified mt-HER2, the kinase-enhance mt-HER2^{V659E} variant strongly induces ROS in mitochondria, increasing the level of constitutive as well as treatment-induced cell death. Unlike mt-HER2, mt-HER2^{V659E} does not stimulate primary tumour growth, despite increasing proliferation in vitro. The faster rate of proliferation might be cancelled out by reduced survival in vivo, as conditions in the primary tumour are suboptimal compared to in vitro settings and mt-HER2^{V659E} cells seem to be sensitised to stress. On the MCF7 background, presence of HER2^{V659E} was incompatible with survival, as we were unable to obtain any mt-HER2^{V659E}-expressing MCF7 clones. These results suggest that the kinase activity of HER2 in mitochondria is pro-tumorigenic but must be regulated to limit ROS-induced cell death and efficiently promote tumour growth.

The limitation of this study is that HER2 was targeted into mitochondria via the N-terminal mitochondrial localisation sequence, which is not the natural way for HER2 to get into the organelle. However, this approach is widely used and is also the best current option for specific mitochondrial targeting. Furthermore, while confirmation in human patient material is warranted, at present there are no tools to selectively manipulate mitochondrial HER2 content. Despite these limitations, our data make a strong case in favour of pro-tumorigenic role of mt-HER2.

AUTHOR CONTRIBUTIONS

Conceptualisation: J.N., K.R., J.R. Methodology: E.N., M.M., D.P., S. M.-N., S.D., J.G., D.Z., T.N., V.F.; Formal Analysis: E.N., M.M., D.P., D.R., M.V., J.R. Investigation: E.N., M.M., D.P., S. M.-N., S.D., K. D., J.G., D.Z., J.H., T.N., V.F. Writing—Original draft: D.P., K.R., J.R.

Writing—Reviewing & Editing: all authors Visualisation: E.N., M.M., D.P. Supervision: D.R., J.P., J.B., J.N., K.R., J.R. Funding acquisition: J.N., K.R., J.R.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

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