# SUPPLEMENTARY INFORMATION

## Receptor Tyrosine Kinase ErbB2 Translocates into Mitochondria and Regulates Cellular Metabolism

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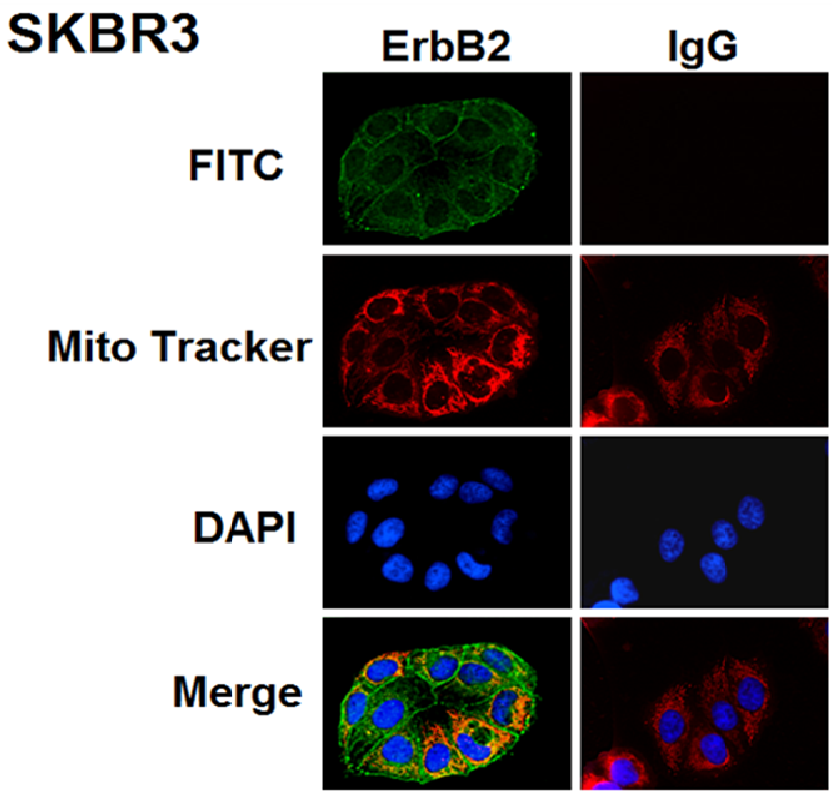
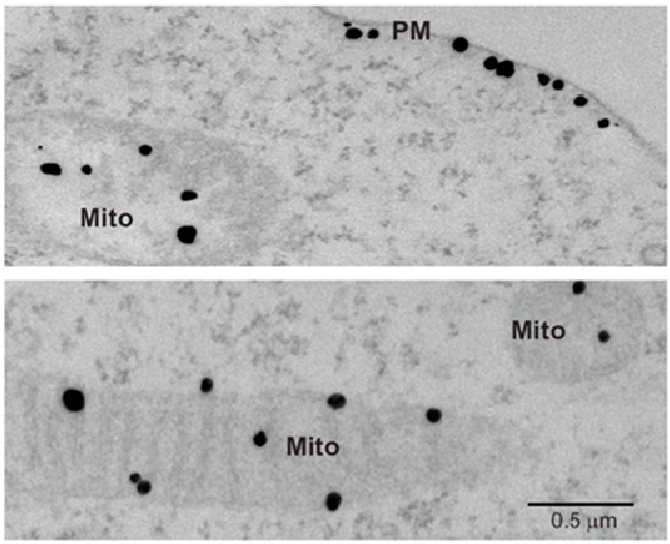
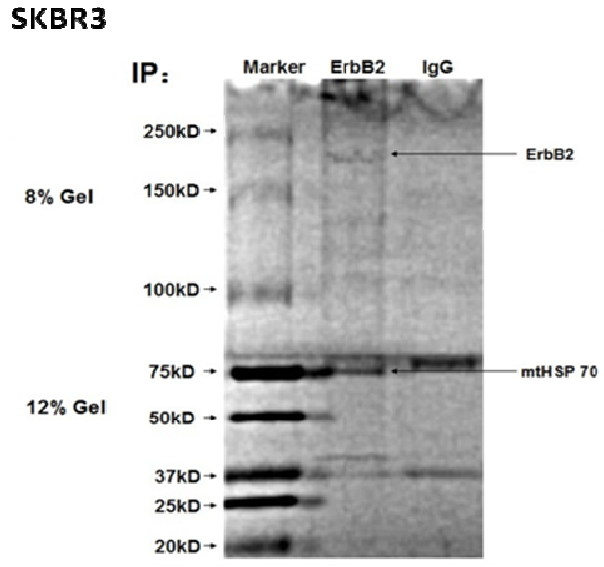
## SUPPLEMENTARY FIGURES



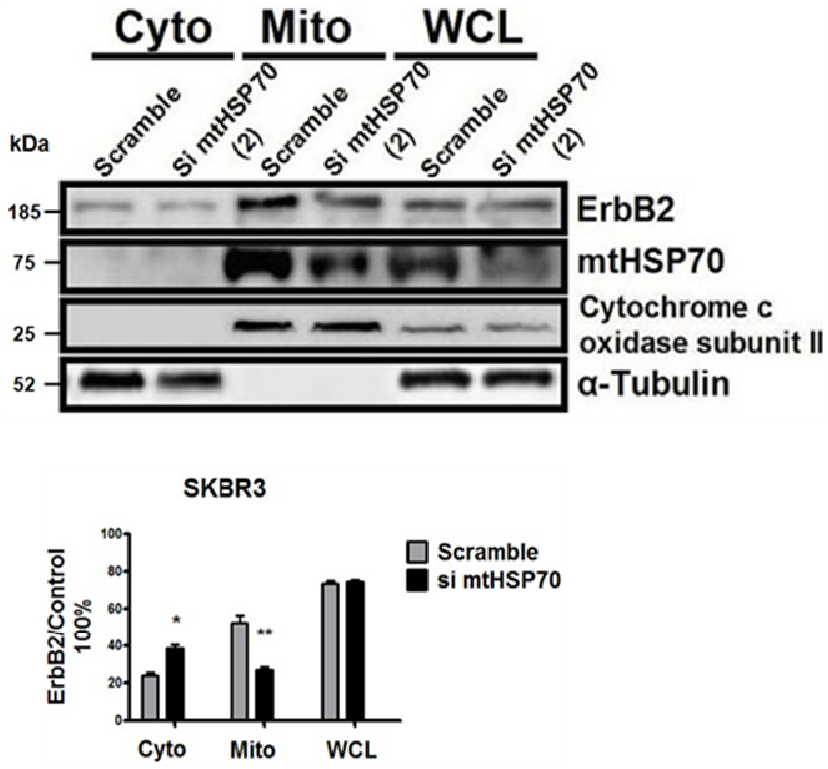

**Supplementary Figure S1. Co-localization of ErbB2 and mitochondria.**
Mitochondria were stained with Mitotracker-Red in SKBR3 cells, cells were fixed and incubated with primary antibody ErbB2 (red), followed by incubation of monoclonal mouse Anti–Cy3 antibody (green) and DAPI (blue). Images were analyzed with Nikon NIS-Elements AR software. Red: mitochondria; Green: ErbB2; Blue: Nuclei; Yellow: Co-localization of ErbB2 and mitochondria. IgG was negative control.



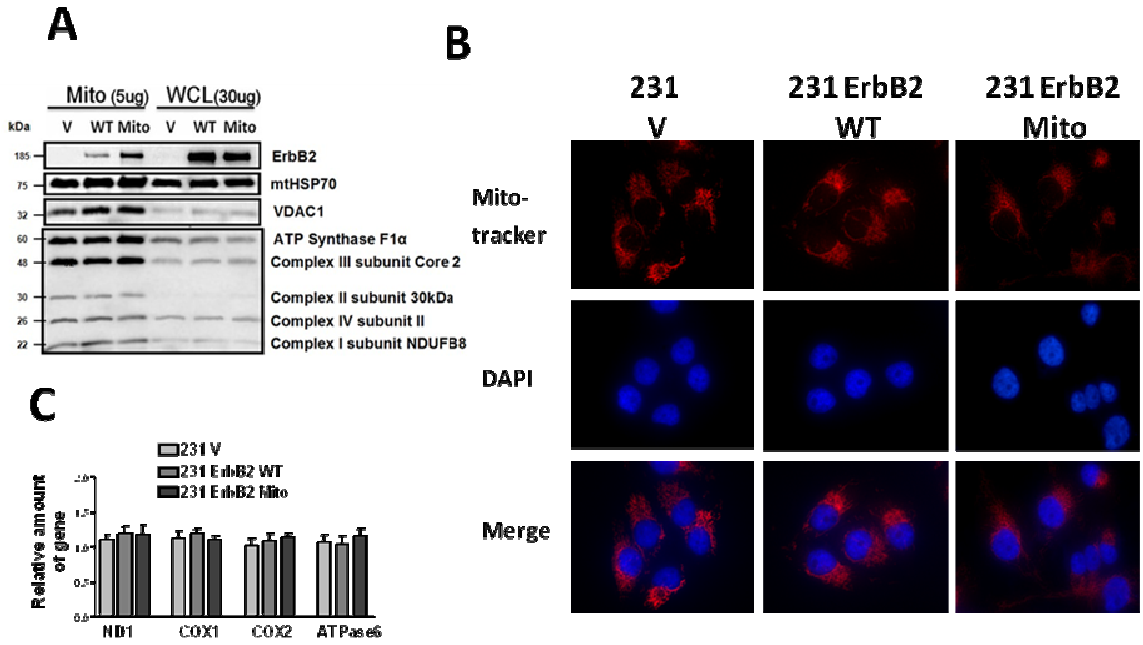

**Supplementary Figure S2**. **Immuno-gold TEM demonstrates mitochondrial localization of ErbB2.** SKBr3 cells were fixed and then incubated with an antibody against ErbB2. Ultrasmall gold particles conjugated to the secondary antibody were silver enhanced for better identification. Two representative images show that ErbB2 located in the mitochondria. Mito: mitochondria, PM: plasma membrane.



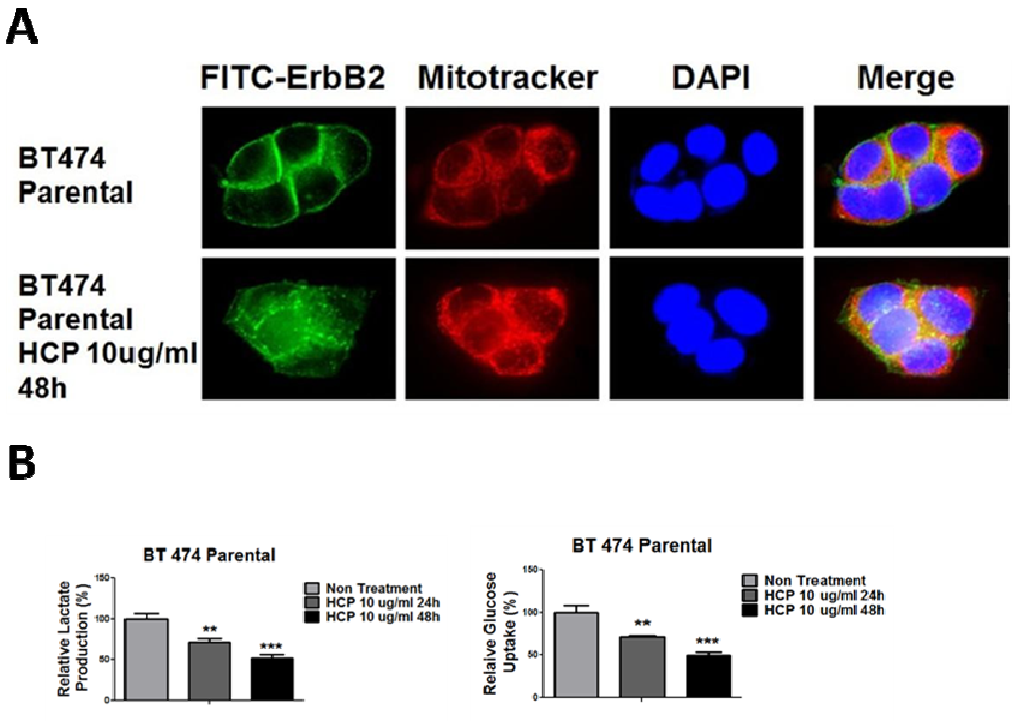

**Supplementary Figure S3**. **Identify ErbB2 binding proteins in the mitochondria of SKBR3 breast cancer cells.** Mitochondrial proteins were isolated from SKBR3 cells and immunoprecipited with ErbB2 antibody. Immunoprecipitates were loaded on a SDS–PAGE followed by Coomassie Blue staining.



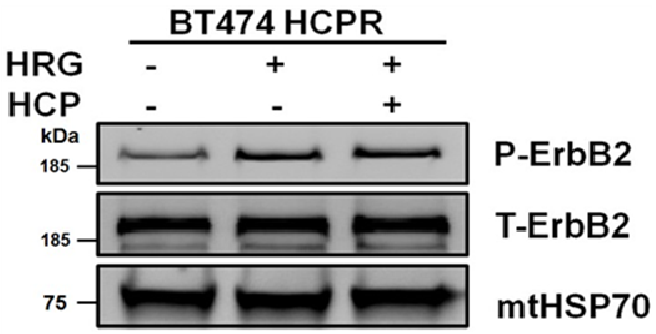

**Supplementary Figure S4**. **Translocation of ErbB2 into mitochondria is facilitated by mtHSP70.** siRNA specific to mtHSP70 was transfected into SKBR3 cells. The cytoplasmic fraction (Cyto), mitochondrial fraction (Mito) and whole cell lysate (WCL) were separated for Western blotting. Cytochrome c oxidase subunit II and α-Tubulin were makers and loading controls for the mitochondrial fraction and the cytoplasmic fraction, respectively.




**Supplementary Figure S5. Mitochondrial integrity of 231V, 231ErbB2WT and 231ErbB2Mito cells.** (A) The expression of components of the ETS were similar in 231V, 231ErbB2WT and 231ErbB2Mito cells. Mitochondrial protein (5 ug) and whole cell lysates (30 ug) were prepared from 231V, 231ErbB2WT and 231ErbB2Mito cells and subjected to SDS-PAGE and probed with the indicated antibodies. mtHSP70 and VDAC1 were used as protein loading controls. (B) The mass of mitochondria in 231V, 231ErbB2WT and 231ErbB2Mito cells did not change significantly. Mitochondria were stained with Mitotracker (red) and nuclei were stained with DAPI (blue). (C) Real time quantitative PCR analysis of mitochondrial DNA-encoded genes in 231V, 231ErbB2WT, and 231ErbB2Mito cells. Total DNA was isolated from 5 $\times 10^6$ cells and analyzed for levels of mitochondrial genes by real-time quantitative PCR. DNA levels of β-Actin were used as an internal control to normalize the amount of mitochondrial genes. The results are presented as relative levels of mitochondrial genes in 231ErbB2WT and 231ErbB2Mito cells compared to 231V cells. Columns, mean of three independent experiments; bars, SE.




**Supplementary Figure S6**. **mtErbB2 contributes to trastuzumab resistance.** (A) Translocation of ErbB2 into mitochondria contributes to Trastuzumab resistance. BT474 breast cancer cells were treated with or without 10 ug/ml Trastuzumab for 48 h. Immunofluorescent staining of ErbB2, mitochondria, and nucleus were performed. Green: ErbB2; red: Mitotracker; blue: nucleus. (B) Trastuzumab inhibits lactate production and glucose uptake in BT474 cells. Columns, mean of three independent experiments; bars, SE.




**Supplementary Figure S7**. **Trastuzumab does not effectively inhibit the activity of mtErbB2 in trastuzumab resistant cells.** BT474 trastuzumab resistant cells were treated without or with Heregulin β1 (HRG), or Heregulin β1 plus Trastuzumab (HCP). Mitochondrial fraction of the cells were isolated and subjected to Western blotting analysis with total ErbB2, p-ErbB2 and mtHSP antibodies.

## SUPPLEMENTARY TABLE

Supplementary Table S1

| Protein | Mitochondria Localization |
|---|---|
| **ErbB2 Full** | **21.7%** |
| **ErbB2 MTS** | **30.4%** |
| **ErbB2 ΔMTS** | **17.4%** |
| **ATP Synthase** | **45%** |
| **IGF1R** | **NP** |
| **Integrin β1** | **NP** |
| **α- Tubulin** | **4.3%** |

NP: Non prediction

Http://psort.ims.u-tokyo.ac.jp/form2.html

**Supplementary Table S1. Mitochondrial Targeting Sequence Prediction.** Mitochondrial Targeting Sequence was predicted by the online program: http://psort.ims.u-tokyo.ac.jp/form2.html, sequences of proteins were obtained from NCBI database.

## SUPPLEMENTARY METHODS

### *Mitochondria preparation and mitochondrial protein isolation*

Intact mitochondria were prepared and purified using the Qproteome Mitochondria Isolation Kit from Qiagen, according to the manufacturer's protocol. Briefly, 3-5x $10^7$ cells were cultured and trypsinized. Washed cells or homogenized tissues were suspended in the Lysis Buffer. Plasma membranes and compartmentalized organelles, such as nuclei, mitochondria, and the endoplasmic reticulum (ER), remained intact and were pelleted by centrifugation. The resulting pellet was resuspended in Disruption Buffer, repeatedly passed through a narrow-gauge needle (to ensure complete cell disruption), and recentrifuged to pellet nuclei, cell debris, and unbroken cells. The supernatant (which contains mitochondria and the microsomal fraction) was recentrifuged to pellet mitochondria. After removal of the supernatant, mitochondria were washed and resuspended in Mitochondria Storage Buffer. For high-purity preparations, the mitochondrial pellet was resuspended in Mitochondria Purification Buffer and carefully pipetted on top of layers of Purification Buffer and Disruption Buffer. During a subsequent centrifugation, mitochondria migrated through the liquid to form a band towards the bottom of the tube. The band was removed and the high-purity mitochondria were pelleted in Mitochondria Storage Buffer.

### *Western Blotting*

Cells were harvested and lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton, 1 mM PMSF and Protease Inhibitor Cocktail (Sigma) for 20 min on ice. Lysates were cleared by centrifugation at 14,000 rpm at 4℃ for 10 min. Supernatants were collected and protein concentrations were determined by the Bradford assay (Bio-rad). The proteins were separated with a SDS/polyacrylamide gel and transferred to a Nitrocellulose membrane (Bio-rad). After blocking in PBS with 5% non-fat dry milk for 1 hr, the membranes were incubated overnight at 4-8℃ with the primary antibodies in PBS with 5% non-fat dry milk. Membranes were extensively washed with PBS and incubated with horseradish peroxidase conjugated secondary anti-mouse antibody or anti-rabbit antibody (1:2,000, Bio-rad). After additional washes

with PBS, antigen-antibody complexes were visualized with the enhanced chemiluminescence kit (Pierce).

### *Immunoprecipitation and mass spectrometry*

Immunoprecipitation experiments were conducted using mitochondrial protein extracted from ErbB2-positive cancer cells. Mitochondrial proteins were isolated and subjected to an immunoprecipitation according to the protocol from the kit. Briefly, an isolated intact mitochondrial fraction was lysed using IP lysis buffer provided from the kit (Pierce Co-immunoprecipitation Kit, #26149) followed by incubation overnight with the primary antibody at 4℃ cold room. After 2 h of incubation with agarose G plus beads, beads were washed and eluted then the elutate was subjected to 10% SDS PAGE gel electrophoreses. The gel was stained with Coomassie Blue and proteins were extracted for Mass Spectrometry analysis.

### *siRNA transfection*

Transfection was performed using the Lipofectamine 2000 (Invitrogen) and following the manufacture's protocol. Thirty-six hours after transfection, cells were lysed and cytoplasmic and mitochondrial fractions were prepared followed by western blotting analysis.

### *Isolation of total DNA and real-time PCR analysis for mtDNA quantification*

DNA isolation and q-RT-PCR analysis for mtDNA quantification were performed according to the method previously reported[40].

### *Apoptosis assay*

The cancer cells were treated with multiple drugs and inhibitors. The late stage of apoptosis was detected by Cell Death Detection ELISA PLUS kit (Roche) according to the manufacturer's instruction.

### *Cell Viability Assay*

A total of $1 \times 10^3$ cells/well were seeded in 96-well plates. Twenty-four hours later, the cell viability was determined by two methods. 1) Using CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to the manufacturer's protocol; 2) by Trypan Blue staining and direct cell counting using a hematocytometer.

## SUPPLEMENTARY REFERENCE

40. Wegrzyn, J. et al. Function of Mitochondrial Stat3 in Cellular Respiration. Science 323, 793 – 797 (2009).