

# Targeting the Mevalonate Pathway to Overcome Acquired Anti-HER2 Treatment Resistance in Breast Cancer



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## Abstract

Despite effective strategies, resistance in HER2<sup>+</sup> breast cancer remains a challenge. While the mevalonate pathway (MVA) is suggested to promote cell growth and survival, including in HER2<sup>+</sup> models, its potential role in resistance to HER2-targeted therapy is unknown. Parental HER2<sup>+</sup> breast cancer cells and their lapatinib-resistant and lapatinib + trastuzumab-resistant derivatives were used for this study. MVA activity was found to be increased in lapatinib-resistant and lapatinib + trastuzumab-resistant cells. Specific blockade of this pathway with lipophilic but not hydrophilic statins and with the N-bisphosphonate zoledronic acid led to apoptosis and substantial growth inhibition of R cells. Inhibition was rescued by mevalonate or the intermediate metabolites farnesyl pyrophosphate or geranylgeranyl pyrophosphate, but not cholesterol. Activated Yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ) and mTORC1 signaling, and their downstream target gene product Survivin, were inhibited

by MVA blockade, especially in the lapatinib-resistant/lapatinib + trastuzumab-resistant models. Overexpression of constitutively active YAP rescued Survivin and phosphorylated-S6 levels, despite blockade of the MVA. These results suggest that the MVA provides alternative signaling leading to cell survival and resistance by activating YAP/TAZ-mTORC1-Survivin signaling when HER2 is blocked, suggesting novel therapeutic targets. MVA inhibitors including lipophilic statins and N-bisphosphonates may circumvent resistance to anti-HER2 therapy warranting further clinical investigation.

**Implications:** The MVA was found to constitute an escape mechanism of survival and growth in HER2<sup>+</sup> breast cancer models resistant to anti-HER2 therapies. MVA inhibitors such as simvastatin and zoledronic acid are potential therapeutic agents to resensitize the tumors that depend on the MVA to progress on anti-HER2 therapies.

## Introduction

The HER2 is amplified and/or overexpressed in about 15% of breast cancers, termed as HER2<sup>+</sup>, where it is a dominant driver of

tumor growth. Effective anti-HER2 treatment with the HER2 mAb trastuzumab combined with chemotherapy has dramatically improved patient outcome (1). Several studies have shown

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that anti-HER2 drug combinations, including the lapatinib + trastuzumab regimen, are even more effective by completely blocking the HER receptor layer (2), and are associated with high rates of pathologic complete response in neoadjuvant clinical trials (3, 4). However, despite the potency of these drug combinations in blocking the HER receptor family, resistance still remains a clinical challenge. Using a panel of HER2<sup>+</sup> breast cancer cell line derivatives made resistant to the lapatinib and lapatinib + trastuzumab regimens, we found that resistance to HER2-targeted therapy may arise from (i) reactivation of the HER2 receptor by various mechanisms including mutations in the HER2 receptor itself, or (ii) activation of escape/bypass pathways such as  $\beta$ -integrin (5, 6) or estrogen receptor (ER; ref. 7) that circumvent anti-HER2 therapy.

The mevalonate pathway is a biosynthetic process regulated by the master transcription factor sterol response element binding protein (SREBP), primarily by SREBP-1a and -2 (8). Cholesterol is the primary end product of this pathway, while isoprenoids, dolichols, sterols, heme A, and ubiquinones are the major intermediate products (Supplementary Fig. S1A). Isoprenoids, particularly farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), play vital roles in a variety of cell processes including cell proliferation, motility, and survival (9). Increasing evidence suggests the important role of the mevalonate pathway in tumor initiation and progression via direct and systemic effects on tumor cells and cells of the immune system (10–13). Upregulation of this pathway promotes mammary cell transformation, and high levels of HMG-CoA-reductase (HMGCR) and other enzymes within this pathway have been shown to correlate with poor survival in breast cancer (14). Similarly, exogenous mevalonate administration promotes tumor growth *in vivo* (12), while blocking this pathway promotes antitumor effects both *in vitro* and *in vivo* (15). ERBB2-dependent upregulation of HMGCR activity has been reported in a HER2<sup>+</sup> breast cancer cell model, supporting the enzyme's potential oncogenic role in this subtype of breast cancer (16). Statins, the commonly used cholesterol-lowering drugs, block the mevalonate pathway by specific inhibition of HMGCR, the rate-limiting enzyme. N-bisphosphonates (including zoledronic acid), another well-known group of mevalonate pathway inhibitors, target the enzyme farnesyl diphosphate synthase (FDPS) and block the formation of the downstream metabolites FPP and GGPP (17). Both statins and bisphosphonates have direct antitumor effects *in vitro* and *in vivo* (15, 18). However, the potential role of the mevalonate pathway in driving resistance to anti-HER2 therapies and the therapeutic potential of mevalonate pathway inhibitors in overcoming this resistance have not been explored.

Yes-associated protein (YAP) and its paralog TAZ (transcriptional coactivator with PDZ-binding motif) function as proto-oncoproteins in a wide variety of cancers and are phosphorylated and inhibited by multiple kinases. YAP and TAZ function as transcriptional coactivators, mainly for the TEAD family of transcription factors, which mediate the oncogenic potential of YAP/TAZ by inducing target genes involved in survival and proliferation (19, 20). Phosphorylation of specific residues on YAP and TAZ results in cytoplasmic sequestration and proteasome-mediated protein degradation (21, 22). In addition, YAP/TAZ activity is regulated by multiple metabolic pathways (23), including the mevalonate pathway, in various cancer cell models (24, 25).

mTOR is a key nutrient, energy, and stress sensor protein that exerts its actions by forming two different complexes (mTORC1

and 2), which can then activate kinases including the S6 kinase and AKT (26). mTOR has been reported to mediate lapatinib resistance in HER2<sup>+</sup> breast cancer (27), although the mechanism remains unclear. *BIRC5*, encoding Survivin, is a member of the inhibitor of apoptosis (*IAP*) gene family, which plays a role in cell survival and the negative regulation of apoptosis (28, 29). Survivin is overexpressed in many cancers, and is associated with chemotherapy resistance and higher tumor recurrence (30). Survivin expression is regulated by various mechanisms including activation of tyrosine kinase receptor signaling (29) and the transcription factor YAP (31).

In this study we sought to assess the role of the mevalonate pathway in mediating anti-HER2 therapy resistance in models wherein HER2 signaling remains inhibited. We uncover the mechanism by which the mevalonate pathway mediates resistance to HER2-targeted treatments. Activation of the pathway provides an alternative proliferation and survival signal to resistant cells, at least partly by activation of the YAP/TAZ transcription factor complex and further downstream mediators Survivin and mTORC1. Finally, we demonstrate the therapeutic potential of mevalonate pathway inhibitors in overcoming this resistance.

## Materials and Methods

Reagents and assays are described in detail in the Supplementary Materials and Methods.

### Cell cultures

The AU565 and UACC812 breast cancer cell lines were purchased from the ATCC. AU565, SKBR3, and UACC812 cells and their cognate lapatinib-resistant, trastuzumab-resistant, and lapatinib + trastuzumab-resistant cells were established and cultured as described in the Supplementary Materials and Methods.

### Western blot analysis

Cells were lysed using RIPA buffer or Cell Signaling Lysis buffer (Cell Signaling Technology), and immunoblotting was performed as described in the Supplementary Materials and Methods. Primary antibodies are listed in the Supplementary Table S1.

### qRT-PCR assay

Primer sequences used are listed in Supplementary Table S2.

### Knockdown by siRNA

Nonspecific control siRNA was Ambion Silencer Select Negative Control #2. Reverse transfection was performed using the Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific). Cell lysates were harvested 72 hours post transfection for protein expression analysis. For cell growth assays, cells were fixed 6 days after transfection. siRNA sequences are listed in Supplementary Table S3.

### Transient transfection of YAP plasmids

XtremeGENE HP Transfection Reagent (Roche) was used for transient transfection per the manufacturer's instructions. The following three YAP plasmids were used: hYAP2 (wild-type) and YAP (S127A) (Addgene plasmids #17793 and #19050, respectively; refs. 32, 33), and YAP (S127/381A) (Addgene plasmid #27378; ref. 21). The plasmid pFLAG-CMV2 was used as a control vector. For transfection experiments using lapatinib-resistant and lapatinib + trastuzumab-resistant cells, the lapatinib and

Sethunath et al.

lapatinib + trastuzumab containing media were replenished 16 to 18 hours after transfection.

### Cell growth assay

Cell growth was analyzed with methylene blue staining. Treatments with the various inhibitors alone or in combination in the presence or absence of the mevalonate pathway metabolites were performed for 6 days. Pretreatments are indicated where applicable. For YAP/TAZ knockdown experiments and YAP overexpression experiments, cells were treated with lapatinib for 6 days, 18 to 48 hours after transfection. Data were normalized to the control treatment within each individual cell derivative from 4 to 8 biological replicates for each treatment group.

### Luciferase reporter assay

Luciferase assays were performed with a well-established SREBP responsive pSRE-luc reporter (34) and a YAP/TAZ-responsive 8XGTTC-luc reporter (Addgene plasmid 34615; refs. 24, 35), respectively. Data were normalized to control treatment within each individual cell derivative from four biological replicates for each treatment group.

### Statistical analysis

For the YAP/TAZ siRNA growth experiment, data analyses were performed using SAS9.4 software for both siRNA combinations (siYT-1 and siYT-2) separately. Cell growth was compared using a general linear model for nonspecific siRNA, siRNA against YAP/TAZ, and their interaction for the parental/lapatinib-resistant/lapatinib + trastuzumab-resistant cell derivatives. For purposes of plotting, model-estimated group means and 95% confidence limits were used. All the other statistical analyses were performed using GraphPad Prism 6.05 software. One-way or two-way ANOVA with Bonferroni multiple comparisons correction was conducted to examine the statistical differences in cell growth with various treatments, in relative light units in luciferase assays, and in mean expression levels of proteins among the groups of interest in reverse phase protein array (RPPA) analysis. All the data in growth, apoptotic, and luciferase assays are presented as mean  $\pm$  SD, except the drug response assays in which data are presented with individual values from four to eight biological replicates. In drug response assays, the drug dose values were log transformed before curve fitting. Curve fitting was performed with a nonlinear variable slope (four-parameter) regression model using the least squares fitting method. LogIC<sub>50</sub> (relative) differences were assessed by the extra sum-of-squares *F* test. Values of *P* < 0.05 were considered to be statistically significant. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001; \*\*\*\*, *P* < 0.0001; and ns, nonsignificant.

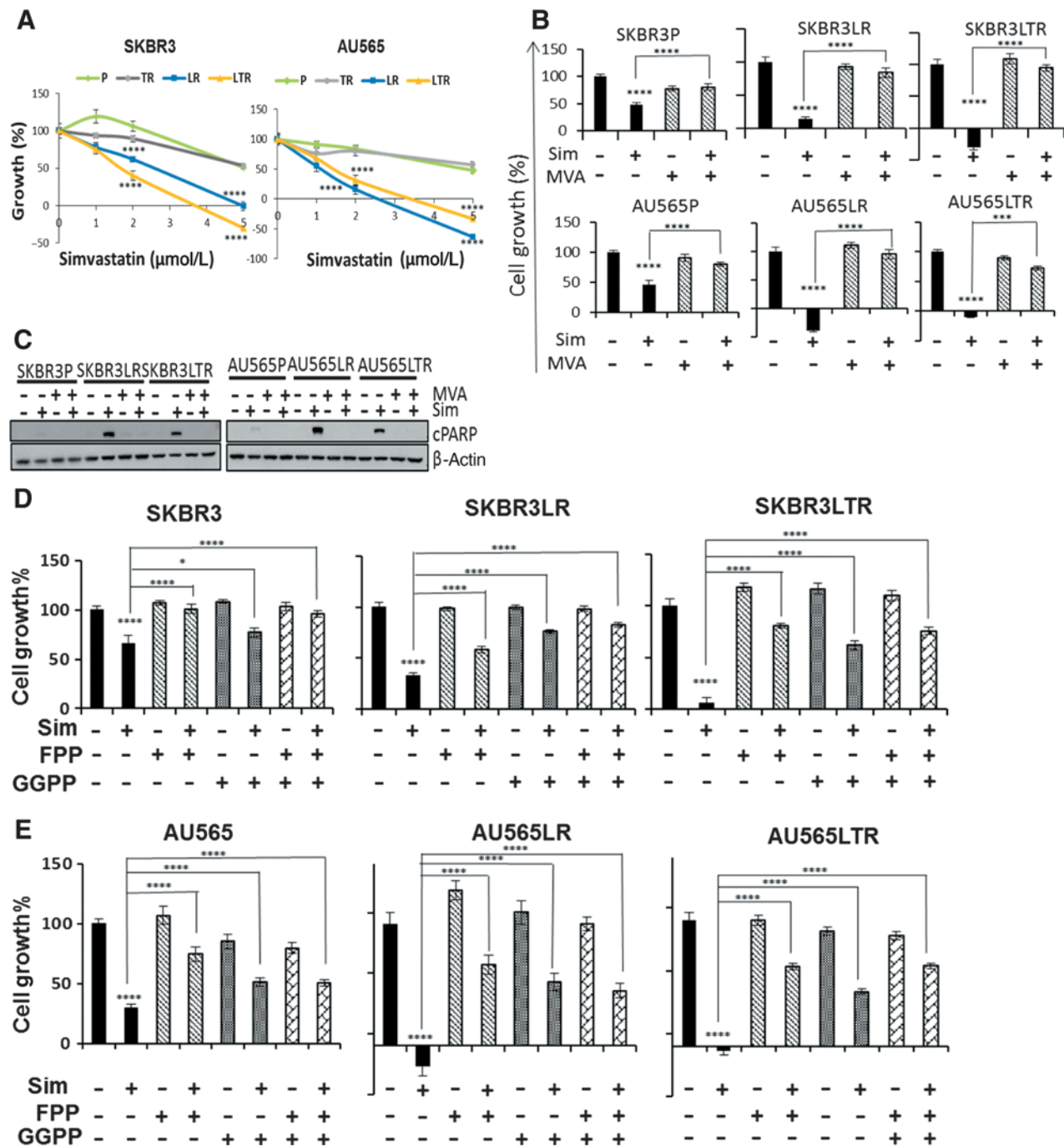
## Results

### Resistant cells are dependent on the mevalonate pathway for cell survival and proliferation

The mevalonate pathway is a complex biosynthetic process comprising multiple enzymatic reactions that produce cholesterol and intermediate isoprenoids (Supplementary Fig. S1A). To investigate whether the mevalonate pathway is involved in acquired anti-HER2 therapy resistance, we used two HER2-amplified (+) cell lines, SKBR3 and AU565, and the HER2<sup>+</sup>/ER-positive cell line UACC812, all of which were initially lapatinib/lapatinib + trastuzumab sensitive, and their lapatinib/lapatinib + trastuzumab-resistant derivatives in which HER signaling

remains substantially inhibited by the continued presence of lapatinib or lapatinib + trastuzumab (Supplementary Fig. S1B; ref. 7). RNA Seq data (uploaded to Gene Expression Omnibus, accession number: GSE132055) from the untreated and treated parental cell lines and their resistant derivatives revealed that the average expression of the key genes in the mevalonate pathway (Supplementary Table S4), as a surrogate for pathway activity, was dramatically suppressed by treatment of parental cells. However, expression was restored or further upregulated relative to parental cells in all three resistant models (Supplementary Fig. S1C). Overall, these analyses suggested that resistant cell growth is associated with restoration of mevalonate pathway expression. Increased expression levels of the mevalonate pathway enzymes were associated with higher SREBP activity, demonstrated by an increase in SRE-responsive luciferase reporter activity in both lapatinib-resistant and lapatinib + trastuzumab-resistant cells (Supplementary Fig. S1D). Overall, these analyses suggested potential involvement of the mevalonate pathway in the resistant phenotype.

Next, to directly examine the mevalonate pathway's role in resistant cell proliferation and survival, the lapatinib-resistant or lapatinib + trastuzumab-resistant derivatives of SKBR3 and AU565 cells together with their parental cells were treated with statins to block the mevalonate pathway. Interestingly, simvastatin, at doses previously reported to have no cytotoxic effect on the immortalized breast cell line MCF-10A (36), dramatically inhibited the growth of lapatinib-resistant and lapatinib + trastuzumab-resistant cells in a dose-dependent manner, and induced cell death (Fig. 1A). On the other hand, the inhibitory effect of simvastatin on the growth of parental and trastuzumab-resistant cells, where the HER2 pathway remains active, was significantly less, suggesting the specificity of the mevalonate pathway in mediating resistant cell growth when HER2 remains inhibited. Another lipophilic statin, atorvastatin, demonstrated a similar growth inhibition of lapatinib-resistant/lapatinib + trastuzumab-resistant versus parental cells (Supplementary Fig. S2A). In contrast, the hydrophilic statin pravastatin had no effect on cell growth (Supplementary Fig. S2B), probably due to the absence of the hepatic tissue-specific transporter required for entry of hydrophilic statins into cells (37, 38). The cell growth inhibitory effect of simvastatin at high and low dosages was significantly reversed (Fig. 1B; Supplementary Fig. S2C) by exogenous supplementation with mevalonate, suggesting that the growth inhibition by simvastatin was via specific blockade of the mevalonate pathway and that the lapatinib-resistant/lapatinib + trastuzumab-resistant cells are highly dependent on this pathway for growth and survival. A selective growth inhibitory effect was also observed using the UACC812 lapatinib + trastuzumab-resistant model (Supplementary Fig. S3A) in which HER2 signaling remains completely inhibited (7). Growth inhibition in this model was also rescued by exogenous mevalonate (Supplementary Fig. S3B). Simvastatin selectively induced apoptosis in the SKBR3-, AU565-, and UACC812-resistant cells, as seen by induced cleaved PARP (c-PARP) protein levels (Fig. 1C; Supplementary Fig. S3C), which was reversed by exogenous mevalonate (Supplementary Fig. S3D). Apoptotic induction was also confirmed by increased annexin V staining (Supplementary Fig. S4). However, we have previously shown that resistance to potent lapatinib + trastuzumab inhibition in HER2<sup>+</sup>/ER<sup>+</sup> cell models including UACC812 lapatinib + trastuzumab resistance is mediated by the ER pathway (7) and, because the mevalonate pathway increases

**Figure 1.**

Lapatinib-resistant or lapatinib + trastuzumab-resistant cells are highly dependent on the MVA pathway for growth and survival. **A**, Cell growth assay with simvastatin (Sim) treatment. Parental (P) SKBR3 or AU565 cells in parallel with their resistant (R) derivatives were treated with increasing doses of simvastatin. Statistical significance levels are indicated for comparisons between parental versus lapatinib resistant (LR) and parental versus lapatinib + trastuzumab-resistant (LTR) models of each cell line. **B**, Cell growth assay with 5  $\mu\text{mol/L}$  simvastatin  $\pm$  250  $\mu\text{mol/L}$  mevalonate (MVA) treatment. **C**, Apoptotic analysis by Western blotting for cPARP. Cells were treated with 5  $\mu\text{mol/L}$  simvastatin  $\pm$  250  $\mu\text{mol/L}$  mevalonate for 48 hours (SKBR3 model) and 72 hours (AU565 model), respectively. Growth assays for simvastatin treatments  $\pm$  FPP or GGPP (10  $\mu\text{mol/L}$ ) of SKBR3 (**D**) or AU565 (**E**) cell models. Values of  $P < 0.05$  were considered to be statistically significant (\*,  $P < 0.05$ ; \*\*,  $P < 0.001$ ; \*\*\*,  $P < 0.0001$ ; TR, trastuzumab resistant).



levels of 25/27-hydroxy-cholesterol that can activate ER as an alternative ligand under estrogen deprivation (39), we focused mainly on ER-negative cell models to avoid this confounding effect.

Importantly, the addition of downstream metabolites of the mevalonate pathway, FPP and GGPP, either alone or in combination rescued cell growth inhibition by simvastatin (Fig. 1D and E). However, neither squalene, the immediate downstream metabolite of FPP, nor its end product cholesterol could rescue the cell growth inhibition conferred by simvastatin treatment (Supplementary Fig. S5A and S5B). Together, these data suggest that the lapatinib and lapatinib + trastuzumab resistance is dependent on the restored mevalonate pathway activity at least partly via its intermediary metabolites FPP and GGPP.

### The mevalonate pathway signals through mTOR and YAP/TAZ to mediate resistance

To identify the key downstream effectors of the mevalonate pathway that facilitate resistance to potent HER2 inhibition, the SKBR3 lapatinib + trastuzumab-resistant cells treated with vehicle, simvastatin, or simvastatin + mevalonate for 24 hours were subjected to RPPA (Supplementary Fig. S6A). We found that simvastatin treatment upregulated the levels of phosphorylated p38 MAPK, JNK, and c-JUN proteins, an effect that was blocked by simvastatin + mevalonate treatment, suggesting that these changes in levels of proteins are associated with mevalonate pathway alterations (Cluster 1; Supplementary Fig. S6B). Several prior studies have demonstrated that simvastatin-induced cell growth inhibition is mediated by p38 MAPK (40) and JNK (41, 42). However, p38 MAPK and JNK inhibitors did not reverse the simvastatin-induced cell growth inhibition in SKBR3 lapatinib-resistant and lapatinib + trastuzumab-resistant cells (Supplementary Fig. S6D), suggesting, either that the growth inhibitory effect of simvastatin is not mediated entirely by either p38 MAPK or JNK, or alternatively, that these protein changes are related to cellular stress due to the simvastatin-mediated growth inhibition.

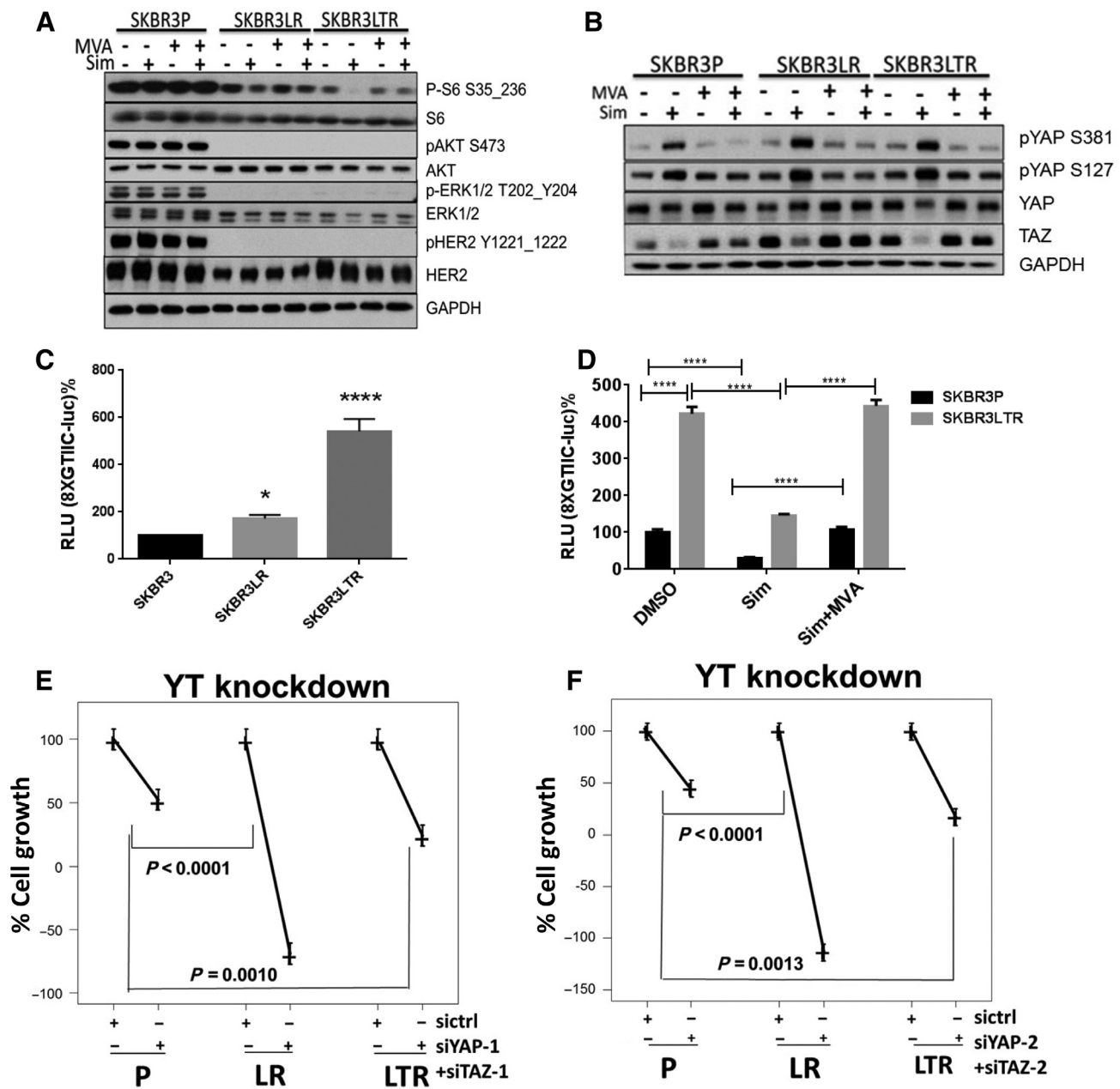
RPPA analysis also showed that levels of several other proteins including YAP and phosphorylated ribosomal protein S6 (p-S6\_S235/236), a surrogate marker for mTORC1 activity, were downregulated by simvastatin treatment and restored by exogenous mevalonate (Cluster 2; Supplementary Fig. S6C). Western blot analysis of the resistant cells revealed that while p-AKT was suppressed in these cells to levels unmeasurable by Western blotting, the levels of p-S6 and p-4EBP1 (p4EBP1\_T37/46) (another marker of mTORC1 activity) were suppressed in parental cells treated with lapatinib or lapatinib + trastuzumab, but increased in lapatinib-resistant and lapatinib + trastuzumab-resistant derivatives of both SKBR3 and AU565 cells (Supplementary Fig. S7A and S7B). On the basis of our findings that mTOR is activated in the resistant cell models and that simvastatin has an inhibitory effect on the levels of p-S6 in these cells, we assessed whether mTOR may be involved in downstream signaling by the mevalonate pathway in the resistant cells. Upon simvastatin treatment, p-S6 levels were decreased in the SKBR3 lapatinib-resistant/lapatinib + trastuzumab-resistant models, but not in the parental cells, and this reduction was completely reversed by exogenous mevalonate (Fig. 2A; Supplementary Fig. S6C), corroborating previous reports that mTOR can be activated in an AKT-independent manner in lapatinib-resistant cells (27, 43). Inhibition of mTORC1 by everolimus inhibited cell growth (Supplementary Fig. S8), indicating that mTORC1 is critical for the resistant growth.

RPPA analysis further identified YAP as one of the key proteins downregulated upon mevalonate pathway blockade, and several recent studies (24, 25) have reported that in various cancers, including breast cancer, the mevalonate pathway regulates YAP/TAZ activity. We then tested whether YAP and TAZ function as downstream effectors of the mevalonate pathway to mediate resistance to HER2-targeted therapy. To further validate the alterations in YAP protein and activity levels observed by RPPA analysis upon pharmacologic blockade of the mevalonate pathway (Supplementary Fig. S5), the SKBR3 parental, lapatinib-resistant, and lapatinib + trastuzumab-resistant cells were treated with simvastatin ± mevalonate for 24 hours. Consistent with the RPPA results, simvastatin treatment dramatically increased the inactive form of p-YAP (S127 or S381) and reduced total YAP/TAZ protein levels, respectively, which were completely restored by simultaneous exogenous mevalonate treatment (Fig. 2B). Of note, simvastatin inhibited YAP/TAZ levels and activity in both parental and resistant cells, although growth was selectively inhibited in the resistant cells. These data further suggest that the mevalonate pathway at least partly regulates YAP/TAZ independent of the growth state of the cells. Inhibitory effects of simvastatin on YAP/TAZ and p-S6 were also reversed by FPP and GGPP (Supplementary Fig. S9). In agreement with the increased levels of the pYAP\_S127 inactive form with simvastatin treatment, immunofluorescence staining showed that YAP protein was localized in the cytoplasm after simvastatin treatment and its nuclear localization was rescued by exogenous mevalonate (Supplementary Fig. S10). In addition, a YAP/TAZ-responsive and TEAD-dependent luciferase reporter (8XGTTC-luc vector) assay showed that YAP/TAZ activity was increased by about 3- and 10-fold in the SKBR3 lapatinib-resistant and lapatinib + trastuzumab-resistant cells, respectively, compared with the parental cells (Fig. 2C). YAP/TAZ transcriptional activity was markedly suppressed by simvastatin but restored by mevalonate (Fig. 2D), and by GGPP plus cholesterol, but not by cholesterol alone (Supplementary Fig. S11). Taken together, these data indicate that YAP/TAZ signaling is elevated in the lapatinib-resistant and lapatinib + trastuzumab-resistant derivatives and is regulated by the mevalonate pathway.

To determine whether the increased YAP/TAZ signaling observed in resistant cells plays a role in cell survival and growth, we performed concurrent knockdown of YAP and TAZ proteins in the SKBR3 parental and resistant cells using two independent sets (siYT-1 and siYT-2) of previously validated YAP and TAZ siRNAs (24, 35). As shown in Supplementary Fig. S12, YAP/TAZ knockdown inhibited the growth of both parental and resistant cells, but the degree of cell growth inhibition upon YAP/TAZ knockdown was significantly greater in the lapatinib-resistant/lapatinib + trastuzumab-resistant cells compared with parental cells (Fig. 2E), suggesting a greater dependence of the resistant cells on the mevalonate pathway-dependent YAP/TAZ signaling.

### mTORC1 is activated through YAP/TAZ in the resistant cells

Having identified both mTORC1 and YAP/TAZ as downstream components of the mevalonate signaling pathway, we investigated their functional relationship. Concurrent knockdown of YAP and TAZ markedly and selectively inhibited the p-S6 levels in both lapatinib-resistant and lapatinib + trastuzumab-resistant but not in parental cells (Fig. 3A; Supplementary Fig. S13A). Ectopic overexpression of constitutively active YAP (S127A, and the double mutant S127/381A) strikingly increased mTORC1 activity in the SKBR3- and AU565-resistant cells, as evidenced by elevated

**Figure 2.**

mTORC1 and YAP/TAZ are downstream effectors of the MVA pathway. **A**, Western blot analysis of HER signaling and its downstream signaling including AKT/mTORC1 and ERK1/2 upon simvastatin (Sim) treatment  $\pm$  mevalonate (MVA) depletion. SKBR3 lapatinib-resistant/lapatinib + trastuzumab-resistant cells in parallel with their parental (P) cells were treated with simvastatin  $\pm$  mevalonate. **B**, Western blotting of YAP/TAZ signaling. SKBR3 parental and the lapatinib-resistant (LR)/lapatinib + trastuzumab-resistant (LTR) derivatives were treated with simvastatin, mevalonate, or simvastatin + mevalonate. **C** and **D**, YAP/TAZ luciferase reporter assay (8XGTTC-luc) for the SKBR3 cell models. Statistical significance levels are indicated for comparison between parental versus lapatinib resistant and parental versus lapatinib + trastuzumab-resistant models. **C**, SKBR3 cell lysates were harvested 24 hours after transfection. Data are presented as relative light units (RLU %) normalized to that of SKBR3P cells. **D**, Cells treated with simvastatin  $\pm$  mevalonate. The data are presented as in **C**. Values of  $P < 0.05$  were considered to be statistically significant. \*,  $P < 0.05$ ; \*\*\*\*,  $P < 0.0001$ . **E** and **F**, Growth assay with YAP/TAZ knockdown. Cells were silenced with two combinations of different siRNA sequences in SKBR3 parental, lapatinib-resistant, and lapatinib + trastuzumab-resistant cells. Data are normalized to the control siRNA group (siCtrl) within individual cell derivatives. **E**, siYT-1, siYAP-1+siTAZ-1; **F**, siYT-2, siYAP-2+siTAZ-2.

p-S6 levels while AKT remained inhibited (Fig. 3B; Supplementary Fig. S13B and S13C), and also reversed the simvastatin-mediated reduction in p-S6 levels (Fig. 3B; Supplementary Fig. S13B and S13C). These results suggest that in the resistant

cells, mTORC1 activity is largely dependent on YAP. In contrast, in the parental cells, overexpression of YAP did not increase p-S6 levels, but partially restored the levels that were reduced by lapatinib treatment, which also blocked AKT and ERK1/2 activity

(Supplementary Fig. S13D). Interestingly, similar to simvastatin, an AKT inhibitor (AZD5363) also reduced p-S6 levels and induced c-PARP, and when combined together, showed an additive effect (Supplementary Fig. S12A and S12B). This suggests that despite partial activation of mTOR by the low residual AKT signaling in resistant cells, mTORC1 is also directly regulated by the mevalonate pathway. Overall, these data suggest that in the lapatinib-resistant and lapatinib + trastuzumab-resistant cells in which HER and downstream AKT signaling remain blocked, reactivation of mTORC1 is largely dependent on the mevalonate pathway via activation of YAP/TAZ. In agreement with this notion, inhibition of p-S6 and induction of c-PARP by simvastatin in the parental cells was observed only in the presence of an AKT inhibitor.

### The antiapoptotic factor Survivin (BIRC5) is a downstream mediator of YAP/TAZ signaling

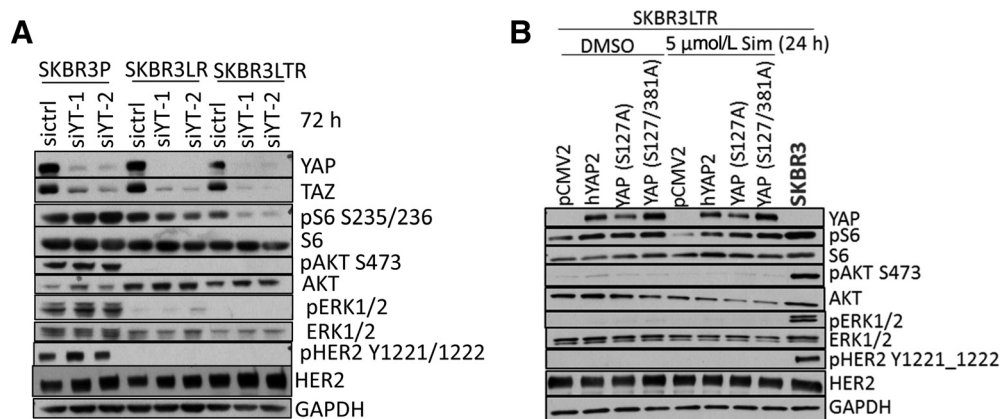
Because YAP/TAZ acts as a transcription factor, we studied the expression levels of several key YAP target genes (*BIRC5*, *CTGF*, and *CYR61*), which are known to promote tumor growth and/or survival (44), in the parental and resistant derivatives of SKBR3 cells. The mRNA expression level of *BIRC5*, encoding Survivin, was downregulated by simvastatin treatment in resistant, but not in parental cells (Fig. 4A), and this inhibition was rescued by exogenous mevalonate. The expression of the other YAP/TAZ target genes (i.e., *CTGF* and *CYR61*), however, was not inhibited by simvastatin, and hence these were excluded from further studies (Supplementary Fig. S15A and S15B). Corresponding to the mRNA expression, we also observed a decrease in Survivin protein levels following simvastatin treatment in the resistant models, but not in the parental cells (Fig. 4B; Supplementary Fig. S15C and S15D). We next attempted to link the regulation of *BIRC5* expression to YAP/TAZ in the resistant cells and observed that *BIRC5* mRNA levels were downregulated upon concurrent YAP/TAZ knockdown (Fig. 4C; Supplementary Fig. S15E). Transient overexpression of the constitutively active YAP mutants in both SKBR3 lapatinib + trastuzumab-resistant cells (Fig. 4D) and AU565 lapatinib + trastuzumab-resistant cells (Supplementary Fig. S16A) increased Survivin levels, and reversed the simvastatin-

mediated inhibition of Survivin expression, suggesting that in the lapatinib + trastuzumab-resistant cells, Survivin expression is largely dependent on mevalonate pathway-mediated YAP/TAZ activation. In both SKBR3 and AU565 cells, we did not observe a decrease in *SLC7A5* (leucine transporter) expression upon inhibition of the mevalonate pathway with simvastatin (Supplementary Fig. S16B and S16C), suggesting that the mechanism by which YAP/TAZ activates mTORC1 in HER2<sup>+</sup> breast cancer cells with acquired resistance to anti-HER2 therapy is different from that reported previously in hepatic epithelial cells (45).

To assess the importance of mTOR in regulating Survivin expression (43) in the resistant models, the SKBR3 parental, lapatinib-resistant, and lapatinib + trastuzumab-resistant cells were treated with low levels of the mTORC1/2 inhibitor AZD2014 and the mTORC1 inhibitor everolimus. mTOR inhibition, especially by AZD2014, suppressed Survivin levels in SKBR3LR and lapatinib + trastuzumab-resistant cells (Supplementary Fig. S16D), indicating that in addition to being directly regulated by YAP/TAZ, Survivin is also regulated by mTOR signaling in the resistant cell models.

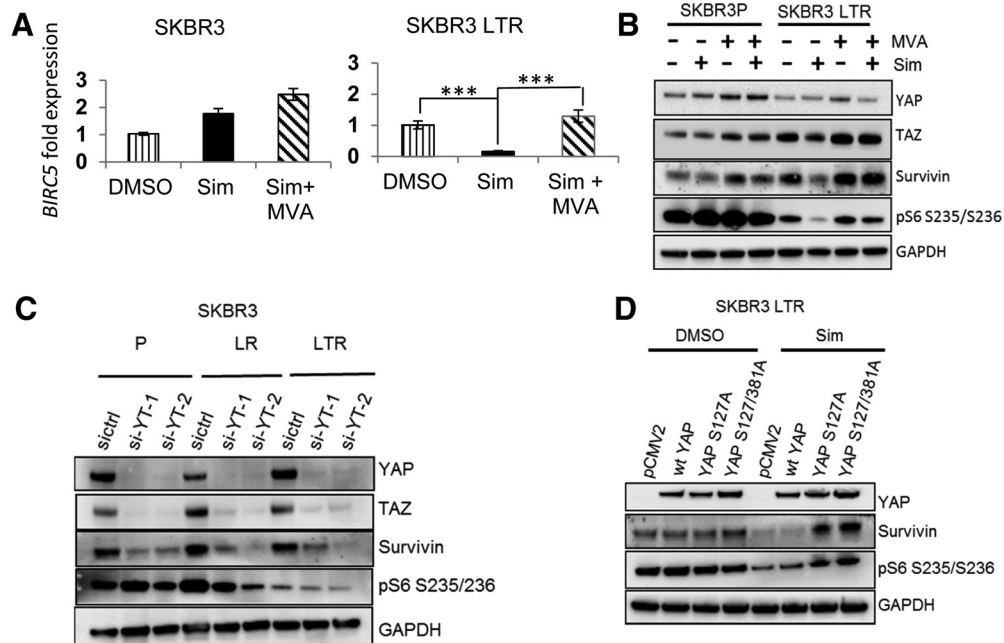
### Inhibition of the mevalonate pathway or its downstream effectors increases the sensitivity of naïve cells to lapatinib

Because the HER2 therapy resistant cells are highly dependent on the mevalonate pathway and its downstream targets for growth and survival, we hypothesized that blockade of the mevalonate pathway or its downstream mediators will further increase the sensitivity of parental cells to lapatinib. Indeed, upon blockade of the mevalonate pathway by simvastatin, the lapatinib dose-response curves of both SKBR3 and AU565 parental cells showed a significant left shift (Fig. 5A). Simvastatin treatment alone had minimal effects on p-HER2\_Y1221/1222, p-AKT\_S473, p-ERK1/2\_T202/Y204, and p-S6 protein levels. Interestingly, in the presence of simvastatin, lapatinib treatment showed a greater reduction in p-S6 levels, with lesser effect on p-HER2\_Y1221/1222, p-AKT\_S473, and p-ERK1/2\_T202/Y204 (Supplementary Fig. S17A and S17B). While the p-S6 level was not affected by lapatinib alone at a low dose of 25 nmol/L, this dose in combination with simvastatin almost completely



**Figure 3.**

mTORC1 is reactivated by YAP/TAZ signaling in resistant cells. **A**, Signaling analysis with YAP/TAZ silenced. Combination knockdown of YAP and TAZ was performed in cells, and cell lysates were collected and assessed by Western blotting for YAP/TAZ levels and HER2 signaling. **B**, Analysis for signaling alterations with YAP ectopic expression in resistant cells under simvastatin (Sim) treatments. After transfection with wild-type (hYAP2) or constitutively active mutant YAP forms [YAP (S127A) and YAP (S127/381A)], SKBR3 lapatinib + trastuzumab-resistant cells were treated with DMSO or 5 μmol/L simvastatin.



**Figure 4.**

Survivin is a downstream mediator of YAP/TAZ signaling in the resistant cells. **A**, *BIRC5* (Survivin) gene expression analysis by qRT-PCR. SKBR3 parental (P) and lapatinib + trastuzumab-resistant (LTR) derivatives were treated with simvastatin (Sim), mevalonate (MVA), or simvastatin + mevalonate. Data were normalized to GAPDH and control treatments of individual cells derivatives. Values of  $P < 0.05$  were considered to be statistically significant; \*\*\*,  $P < 0.001$ . **B**, Western blotting of YAP/TAZ and Survivin protein levels. SKBR3 parental and the lapatinib + trastuzumab-resistant derivatives were treated with simvastatin, or simvastatin + mevalonate. **C**, Signaling analysis with YAP/TAZ silenced. Combination knockdown of YAP and TAZ was performed in SKBR3 parental, lapatinib-resistant (LR), and lapatinib + trastuzumab-resistant cells, and cell lysates were subjected to Western blot analysis for YAP/TAZ, Survivin, and p-S6 protein levels. **D**, Analysis for signaling alterations with YAP ectopic expression in resistant cells under simvastatin treatments. Forty-eight hours after transfection with wild-type (hYAP2) or mutant YAPs [YAP (S127A) and YAP (S127/381A)], SKBR3LTR cells were treated with DMSO or simvastatin.

suppressed p-S6 levels, suggesting that mevalonate pathway blockade enhances the efficacy of lapatinib in abrogating mTORC1 signaling. To assess whether YAP/TAZ signaling plays a similar key role in modulating the lapatinib response, we knocked down YAP/TAZ or overexpressed a constitutively active YAP (S127/381A) mutant in parental (lapatinib/lapatinib + trastuzumab sensitive) cells in the presence of lapatinib. Interestingly, YAP/TAZ knockdown shifted the lapatinib dose-response curve of these cells significantly to the left (Fig. 5B), while overexpression of YAP (S127/381A) significantly reduced their sensitivity to lapatinib (Supplementary Fig. S18A–S18C) and the dual HER1/2 TKI afatinib (Supplementary Fig. S18D), suggesting that YAP/TAZ signaling modulates the cellular response to both TKIs. To investigate whether the ability of simvastatin to enhance sensitivity of cells to lapatinib is because of enhanced inhibition of mTORC1, the lapatinib response was assessed  $\pm$  the mTORC1 inhibitor everolimus in AU565 and SKBR3 parental cells. As shown in Fig. 5C, everolimus rendered both the AU565 and SKBR3 parental cells more sensitive to lapatinib, suggesting that mTORC1 inhibition substantially enhances lapatinib efficacy.

#### The N-bisphosphonate zoledronic acid effectively inhibits resistant cell growth

Finally, we investigated whether acquired HER2-targeted therapy resistance could also be overcome by zoledronic acid, a clinically important mevalonate pathway inhibitor that blocks the enzymatic activity of FDPS (Supplementary Fig. S1A). Like

simvastatin, zoledronic acid greatly inhibited SKBR3, AU565, and UACC812 resistant cell growth, with only modest effects on parental cells (Fig. 6A; Supplementary Fig. S19A). This growth inhibition by zoledronic acid was rescued by GGPP, a metabolite downstream of FDPS, but not by the upstream metabolite mevalonate, demonstrating that zoledronic acid's inhibitory effect on resistant growth is via mevalonate pathway blockade (Fig. 6B; Supplementary Fig. S19B). Furthermore, zoledronic acid induced a greater apoptotic response in the resistant versus parental models, as seen by increased c-PARP which was again reversed by the addition of GGPP (Fig. 6C). Like simvastatin, zoledronic acid increased levels of the inhibitory p-YAP, and decreased levels of total TAZ, p-S6, and Survivin (Fig. 6D), which were rescued by GGPP. Together, these results demonstrate that zoledronic acid exploits a similar mechanism as simvastatin to suppress the growth and survival of cells resistant to anti-HER2 therapy.

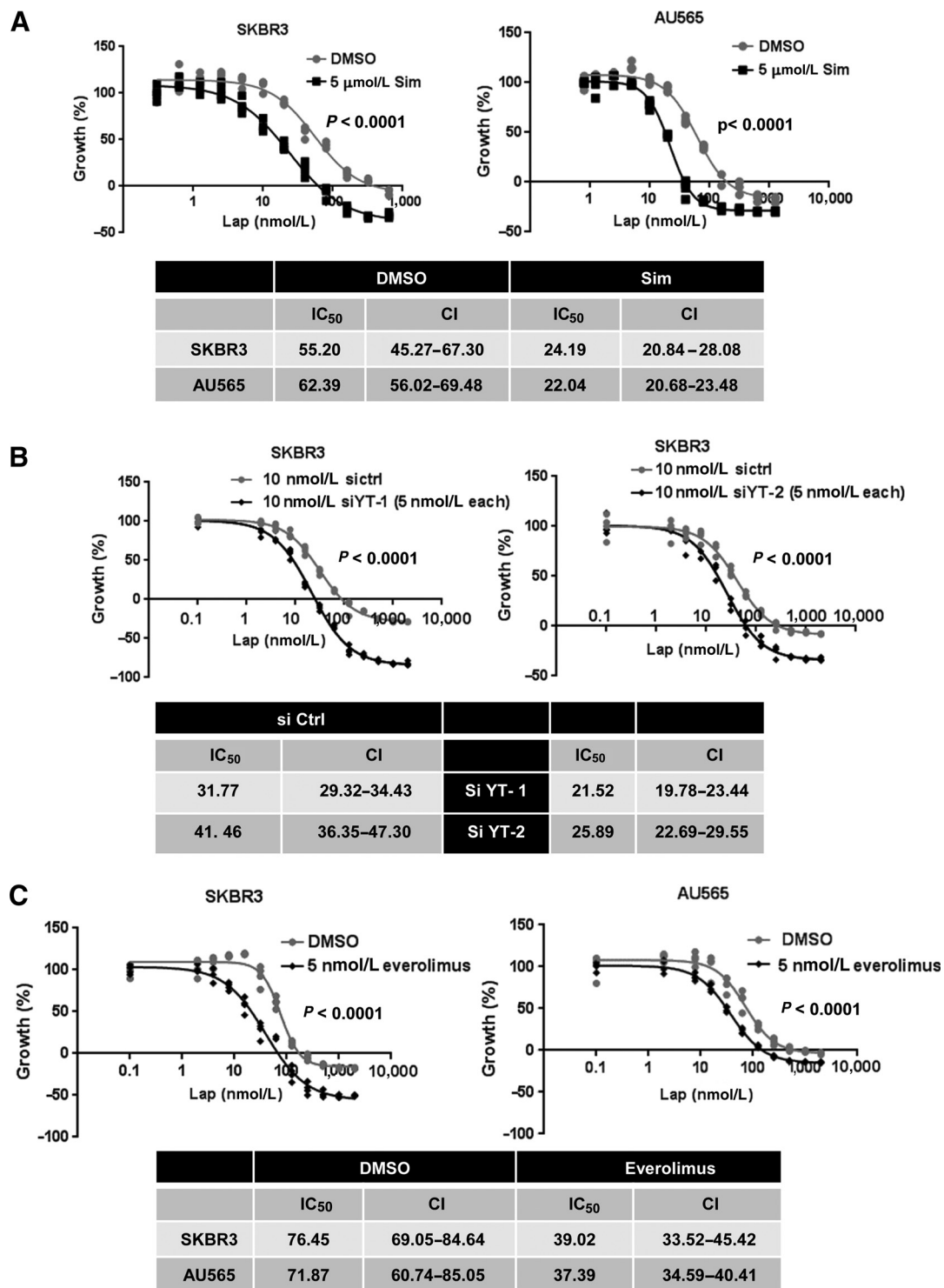
Finally, withdrawal of lapatinib and lapatinib + trastuzumab from the culture media of the resistant cells, led to reactivation of HER2 signaling and reduced sensitivity to both simvastatin and zoledronic acid (Supplementary Fig. S20). This suggests that sustained inhibition of HER2 signaling is indeed required for the mevalonate pathway to act as an escape mechanism of resistance.

## Discussion

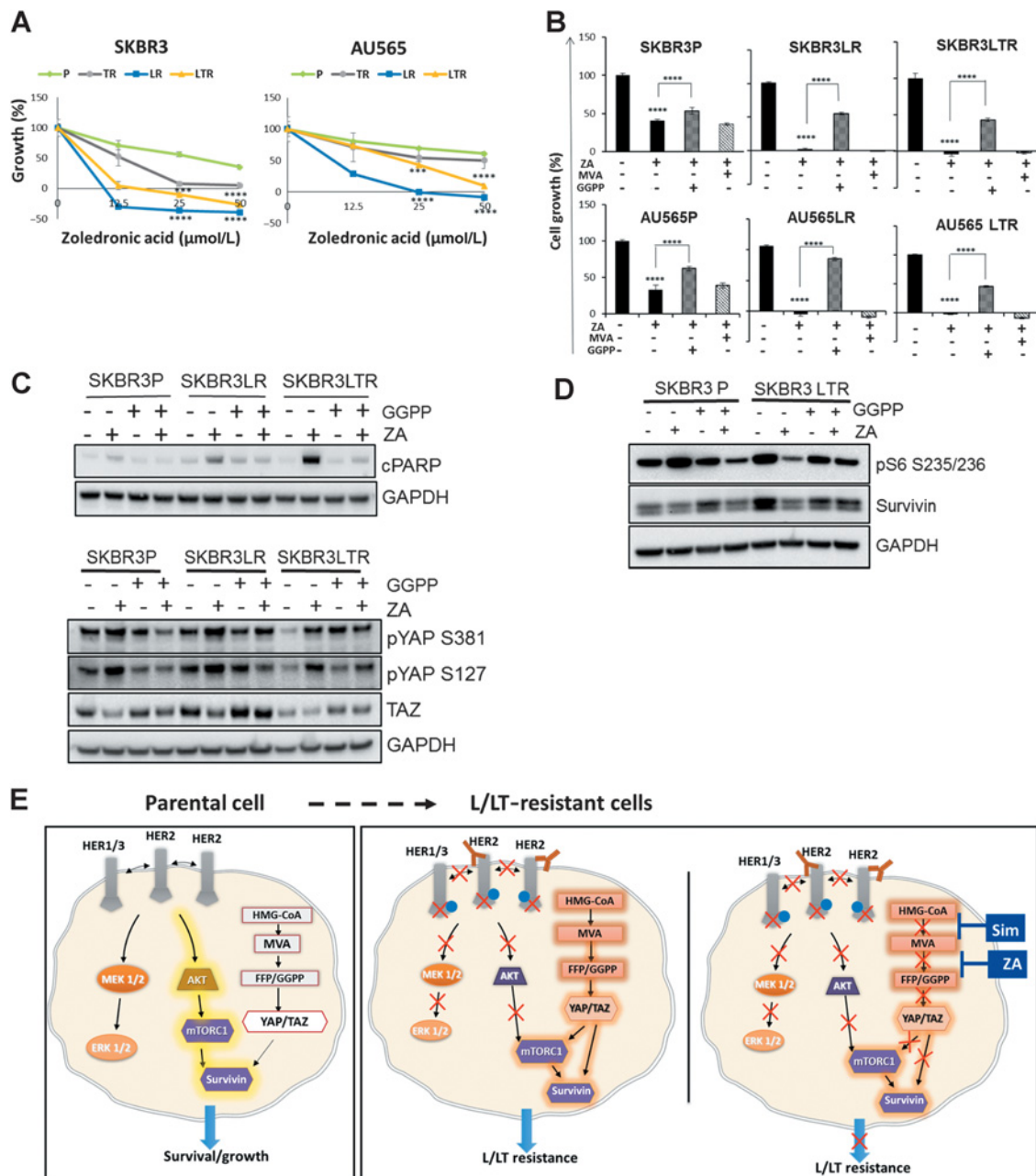
Despite the advent of multiple therapies targeting HER2, resistance is still common and remains a challenge. Using models of



Sethunath et al.

**Figure 5.**

Blockade of the mevalonate pathway and its downstream effectors YAP/TAZ and mTOR enhances lapatinib efficacy. **A**, Lapatinib response assays under mevalonate pathway blockade. SKBR3 or AU565 parental cells were treated with multiple doses of lapatinib  $\pm$  simvastatin. Data were normalized to the mean of non-lapatinib treatments within the DMSO or simvastatin group, respectively. **B**, Growth assays for lapatinib (Lap) response under YAP/TAZ silencing. The SKBR3 parental cells were transfected with siCtrl or siYT-1 siRNAs, and were treated with multiple doses of lapatinib for 5 days. Data are normalized to non-lapatinib treatment within each siRNA. **C**, Lapatinib response assays under mTORC1 suppression. SKBR3 or AU565 cells were treated with lapatinib  $\pm$  5 nmol/L everolimus. Data are presented as in **A**. *P* values are indicated for comparisons between logIC<sub>50</sub> values of control versus treated curves.

**Figure 6.**

Mevalonate pathway blockade by zoledronic acid (ZA) is effective in overcoming lapatinib/lapatinib + trastuzumab resistance by a mechanism similar to simvastatin (Sim). **A**, Cell growth assay with zoledronic acid treatment. SKBR3 or AU565 parental (P) cells in parallel with their lapatinib-resistant (LR), trastuzumab-resistant (TR), and lapatinib + trastuzumab-resistant (LTR) derivatives were treated with 12.5, 25, and 50  $\mu\text{mol/L}$  zoledronic acid. Statistical significance levels are indicated for comparisons between parental versus lapatinib-resistant and parental versus lapatinib + trastuzumab-resistant models of each cell line. **B**, Cell growth assay with 20  $\mu\text{mol/L}$  or 50  $\mu\text{mol/L}$  zoledronic acid (in SKBR3 and AU565 cell models, respectively)  $\pm$  GGPP treatment. For **A** and **B**, values of  $P < 0.05$  were considered to be statistically significant. \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ . **C**, Western blotting for the apoptotic marker c-PARP and YAP/TAZ signaling. c-PARP, pYAPS127, pYAPS397, and TAZ were probed after SKBR3 cells were treated with zoledronic acid  $\pm$  GGPP for 48 hours. **D**, Western blotting of pS6 and Survivin levels. SKBR3 P and the lapatinib + trastuzumab-resistant derivatives were treated with zoledronic acid, GGPP, or zoledronic acid + GGPP for 36 hours for P-S6 and Survivin levels. **E**, Working model presenting the alternative signaling in lapatinib-resistant or lapatinib + trastuzumab-resistant cells where HER signaling remains inhibited by sustained lapatinib/lapatinib + trastuzumab treatment. Parental cells treated with lapatinib/lapatinib + trastuzumab concentrations that substantially inhibit HER signaling and the downstream AKT/mTORC1 and ERK1/2 pathways undergo growth inhibition or cell death. To evade the HER signaling suppression, cells adopt the mevalonate pathway as an alternative pathway to survive. The mevalonate pathway activates downstream YAP/TAZ-mTORC1 and YAP/TAZ-Survivin signaling through FPP/GGPP by unknown mechanisms. The mevalonate pathway inhibitors (simvastatin and zoledronic acid) block the mevalonate pathway signals and help to overcome resistance when HER2 signaling remains blocked due to presence of lapatinib or lapatinib + trastuzumab. The red X's represent blockade of the HER/AKT pathway and the mevalonate pathway.

HER2<sup>+</sup> breast cancer cell lines made resistant to HER2-targeted therapies, we observed that resistance is associated either with reactivation of HER2 signaling, or with alternative signaling pathways that promote cell survival and growth when HER2 signaling is blocked. Using the dual HER1/2 tyrosine kinase inhibitor lapatinib, alone or in combination with trastuzumab, we now report a novel mechanism, representative of the latter method, by which HER2<sup>+</sup> breast cancer cells escape sustained HER2 inhibition. The lapatinib-resistant and lapatinib + trastuzumab-resistant cells in which HER signaling is completely blocked have adopted the mevalonate pathway as an escape route to promote resistant cell growth. This resistance involves activation of and increased dependence on the mevalonate pathway–YAP/TAZ–mTORC1–Survivin signaling axis, identifying a metabolic vulnerability that can be exploited pharmacologically by inhibiting the mevalonate pathway to overcome resistance, as illustrated in the working model presented in Fig. 6E.

Statins and the amino-(N)-bisphosphonate zoledronic acid, which are used frequently in patients to lower blood cholesterol and improve bone density, respectively, specifically block the mevalonate pathway. We observed that while lipophilic statins, which can penetrate the cell membrane, were cytotoxic to the resistant cells, the hydrophilic statins were not, probably due to absence of the transporter that is required for entry of hydrophilic statins into the cells (37, 46). Importantly, in breast cancer, the majority of studies including a recent meta-analysis suggest that lipophilic but not hydrophilic statins have great protective effects and significantly reduce cancer-specific and all-cause mortality in patients with breast cancer (47). Our findings also showed that zoledronic acid was as effective as simvastatin in killing the resistant cells. We demonstrated that inhibition of the mevalonate pathway by both drugs can overcome resistance to anti-HER2 therapy, by inhibiting key downstream survival signaling, when HER2 signaling remains blocked. This selective and specific action of mevalonate pathway inhibitors in circumventing resistant growth of HER2<sup>+</sup> breast cancer cells presents a novel therapeutic application of these inhibitors in breast cancer. We also showed that concomitant treatment of parental cells with these mevalonate pathway inhibitors may enhance the antitumor efficacy of anti-HER2 inhibitors, thereby offering additional therapeutic intervention.

YAP has been shown to mediate resistance to targeted therapies in several reports. Indeed, amplification or overexpression of YAP enables pancreatic, colon, and lung cancers to bypass oncogenic KRAS addiction (48, 49). In this study, we demonstrated that knockdown of YAP/TAZ leads to significantly greater growth inhibition in lapatinib-resistant/lapatinib + trastuzumab-resistant cells than in the parental cells, which have intact HER2 signaling. Conversely, hyperactivation of YAP/TAZ by overexpression of constitutively active YAP attenuated the sensitivity of HER2<sup>+</sup> breast cancer cells to anti-HER2 therapy, thus establishing the role of YAP/TAZ signaling as an escape mechanism that can drive resistance in HER2<sup>+</sup> breast cancer. While YAP/TAZ itself was previously reported to increase microenvironment rigidity, hence mediating lapatinib resistance (50), our study demonstrates its key intermediary role to convey resistance to potent HER2 inhibition or to negate the antitumor effects of HER2-targeted therapy. In line with previous reports about mevalonate pathway-mediated activation of YAP/TAZ (24, 51), our data demonstrated the role of the isoprenoid metabolites, FPP and GGPP, in the mevalonate pathway signaling to YAP/TAZ. The observation that

addition of FPP and GGPP rescues resistant cell growth and YAP/TAZ signaling inhibition by mevalonate pathway inhibitors, whereas squalene and cholesterol could not, confirmed that it is indeed the intermediate isoprenoids which mediate signaling to YAP/TAZ.

In this study, we found that reactivated mTORC1 signaling in anti-HER2-resistant cells, where HER2 remains inhibited, is a potential downstream effector that is upregulated at least partly by the mevalonate pathway via activation of YAP/TAZ. By overexpressing constitutively active forms of YAP, we showed that YAP overcomes simvastatin-mediated inhibition of mTORC1 signaling, suggesting that mTORC1 signaling is directly regulated by the mevalonate pathway and YAP/TAZ. Although previous studies (27, 43) have shown that PI3K-independent activation of mTORC1 confers lapatinib resistance, the underlying mechanisms of activation remained unclear. In this regard, our findings present a novel mechanism for AKT-independent activation of mTORC1 signaling via the mevalonate pathway–YAP/TAZ axis. Of note, although our data suggests that mTOR reactivation is largely dependent on the mevalonate pathway–YAP/TAZ signaling, we found that inhibition of the low residual levels of AKT activity, which could be detected only in the presence of an AKT inhibitor, further inhibited the mTOR activity. We surmise that this residual AKT activity may be attributed to the fact that mTOR is both an effector and activator of AKT signaling (52), or to alternative upstream signaling from the mevalonate pathway or other cellular kinases. Nevertheless, our data suggests that the combination of a mevalonate pathway inhibitor and an AKT inhibitor may be even more effective in overcoming anti-HER2 therapy resistance.

While exploring the downstream survival effectors of mevalonate pathway signaling through YAP/TAZ, we also found that Survivin, an antiapoptotic protein that is a direct gene target of YAP, is regulated by the mevalonate pathway in resistant cells. In addition to its regulation by the mevalonate pathway and YAP/TAZ, we also observed inhibition of Survivin levels by mTORC1 inhibitors. This observation is in agreement with a previous report showing mTOR-mediated inhibition of Survivin in a lapatinib-resistant cell line (43), suggesting that Survivin may also be regulated by mTORC1. Together, these findings imply that Survivin may be a key signaling junction that is responsive to multiple upstream stimuli in the cells resistant to anti-HER2 therapy.

In summary, this study highlights the role of the mevalonate pathway as a novel mechanism of resistance to anti-HER2 therapies in HER2<sup>+</sup> breast cancer cells when HER2 signaling remains inhibited. We identified and linked new targets of the mevalonate pathway, especially the YAP/TAZ–mTORC1–Survivin axis that could be further therapeutically exploited to reverse resistance to HER2-targeted therapies. Our findings warrant additional research to directly assess the potential impact of statins and N-bisphosphonates on HER2<sup>+</sup> breast cancer recurrence, as well as prospective validation in clinical trials to evaluate the potential additive or synergistic effects of statins when used with anti-HER2 therapies. Furthermore, while new antibodies and more potent drugs against HER2 are being developed in the clinic, it will be important to understand the activity status of the HER receptor layer in the residual disease. Our recently launched SIMPHONY trial (NCT03324425), for patients with metastatic HER2<sup>+</sup> breast cancer who progressed on dual anti-HER2 therapy, will evaluate whether the addition of simvastatin to a dual anti-HER2 regimen

can resensitize the tumors to anti-HER2 therapy. If clinically validated, our findings may help develop a new well-tolerated treatment approach.

### Disclosure of Potential Conflicts of Interest

J.S. Reis-Filho is a consultant at Goldman Sachs, is an ad hoc scientific advisory board member at Roche Tissue Diagnostics, Genentech, and Invivo, and is a scientific advisory board member at Volition Rx. M.F. Rimawi is a scientific consultant at Genentech, Daiichi Sankyo, MacroGenics, and Novartis, and reports receiving a commercial research grant from Pfizer. J.W. Gray is an advisor at New Leaf Ventures, reports receiving a commercial research grant from PDX Pharmaceuticals, Quantitative Imaging, has ownership interest (including patents) in PDX Pharmaceuticals and Convergent Genomics, and has provided expert testimony for University of California. C.K. Osborne has received speakers bureau honoraria from SABCS, and has consultant/advisory board relationships with Novartis, AstraZeneca, Lilly, and Tolmar. R. Schiff is a Margetuximab advisory council member at MacroGenics, and reports receiving a commercial research grant from AstraZeneca, PUMA Biotechnology, and Gilead Sciences. No potential conflicts of interest were disclosed by the other authors.

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**Study supervision:** R. Schiff

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