Targeting C4-Demethylating Genes in the Cholesterol Pathway Sensitizes Cancer Cells to EGF Receptor Inhibitors via Increased EGF Receptor Degradation

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ABSTRACT
Persistent signaling by the oncogenic EGF receptor (EGFR) is a major source of cancer resistance to EGFR targeting. We established that inactivation of 2 sterol biosynthesis pathway genes, SC4MOL (sterol C4-methyl oxidase-like) and its partner, NSDHL (NADP-dependent steroid dehydrogenase-like), sensitized tumor cells to EGFR inhibitors. Bioinformatics modeling of interactions for the sterol pathway genes in eukaryotes allowed us to hypothesize and then extensively validate an unexpected role for SC4MOL and NSDHL in controlling the signaling, vesicular trafficking, and degradation of EGFR and its dimerization partners, ERBB2 and ERBB3. Metabolic block upstream of SC4MOL with ketoconazole or CYP51A1 siRNA rescued cancer cell viability and EGFR degradation. Inactivation of SC4MOL markedly sensitized A431 xenografts to cetuximab, a therapeutic anti-EGFR antibody. Analysis of Nsdhl-deficient Bpa2+/− mice confirmed dramatic and selective loss of internalized platelet-derived growth factor receptor in fibroblasts, and reduced activation of EGFR and its effectors in regions of skin lacking NSDHL.

SIGNIFICANCE: This work identifies a critical role for SC4MOL and NSDHL in the regulation of EGFR signaling and endocytic trafficking and suggests novel strategies to increase the potency of EGFR antagonists in tumors. Cancer Discov; 3(1); 1–16. © 2012 AACR.

INTRODUCTION
The EGF receptor (EGFR) provides essential growth and prosurvival signals to epithelial cells and is often targeted therapeutically in epithelial malignancies. Unfortunately, EGFR-antagonizing treatment strategies are often limited in their efficacy due to acquired or primary resistance in tumors. Receptor switching or compensatory activation of EGFR coreceptors such as the receptor tyrosine kinases (RTK) ERBB2 (1), ERBB3 (2), and insulin-like growth factor I receptor (3) are examples of such mechanisms that can interfere with the efficacy of EGFR antagonists in tumors.

RESONS
Persistent signaling by the oncogenic EGF receptor (EGFR) is a major source of cancer resistance to EGFR targeting. We established that inactivation of 2 sterol biosynthesis pathway genes, SC4MOL (sterol C4-methyl oxidase-like) and its partner, NSDHL (NADP-dependent steroid dehydrogenase-like), sensitized tumor cells to EGFR inhibitors. Bioinformatics modeling of interactions for the sterol pathway genes in eukaryotes allowed us to hypothesize and then extensively validate an unexpected role for SC4MOL and NSDHL in controlling the signaling, vesicular trafficking, and degradation of EGFR and its dimerization partners, ERBB2 and ERBB3. Metabolic block upstream of SC4MOL with ketoconazole or CYP51A1 siRNA rescued cancer cell viability and EGFR degradation. Inactivation of SC4MOL markedly sensitized A431 xenografts to cetuximab, a therapeutic anti-EGFR antibody. Analysis of Nsdhl-deficient Bpa2+/− mice confirmed dramatic and selective loss of internalized platelet-derived growth factor receptor in fibroblasts, and reduced activation of EGFR and its effectors in regions of skin lacking NSDHL.

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RESULTS
Probing the Sterol Biosynthesis Pathway for Regulation of Response to EGFR Inhibitors
To address the mechanism of SC4MOL regulation of response to the EGFR inhibitors erlotinib and cetuximab, we

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).
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doi: 10.1158/2159-8290.CD-12-0031
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first considered whether this effect was general to proteins operating in the cholesterol biosynthesis pathway (Fig. 1A) or more specific. SC4MOL is highly conserved throughout evolution, as are many genes operating upstream and downstream in the sterol synthesis pathway (11). Three human catalytic enzymes, SC4MOL, NSDHL, and HSD17B7, and a gene with unknown function, C14ORF1, are orthologous to a complex of yeast C4-sterol demethylation genes that define the “ergosome” (ERG25/SC4MOL, ERG26/NSDHL, ERG27/HSD17B7, and ERG28/C14ORF1; ref. 12).

Depletion of SC4MOL and NSDHL by multiple siRNA or short hairpin RNA (shRNA; Fig. 1B and Supplementary Fig. S1A–S1C), but not of 7 other proteins (SQLE, LSS, CYP51A1, TM7SF2, LBR, HSD17B7, and C14ORF1) operating farther upstream or downstream in the pathway (Supplementary Fig. S1D–S1F), sensitized A431 cells to the EGFR kinase inhibitor erlotinib, suggesting a specific block focused at the C4-demethylation step in the pathway. Similar results were obtained in the head and neck squamous carcinoma cell lines SCC61 (Fig. 1C) and SCC68 (Fig. 1D) expressing moderate levels of EGFR (Supplementary Fig. S2A), and in the lung adenocarcinoma cell line PC9 (Fig. 1E), which expresses a mutated form of EGFR, ΔE746-A750 (13), indicating the findings were not specific to A431 cells. Sensitization was also observed with 2 shRNA constructs targeting SC4MOL (Supplementary Fig. S1C) and was associated with marked enhancement of apoptosis (Supplementary Fig. S2B and S2C). In contrast to sensitization, inactivation of SC4MOL and NSDHL did not affect intrinsic cell growth of the EGFR-high A431 cells made deficient in SC4MOL and NSDHL were treated with indicated inhibitors at concentrations producing 20% to 30% decrement in viability. In B–F, viability data from 3 independent experiments were normalized to mock-treated GL2 control-transfected cells. Each column represents averaged results; bars, SDs. *P < 0.001.
Sterol Pathway Genes Regulate Tumor Response to Anti-EGFR

Targeting SC4MOL and NSDHL specifically sensitized cells to erlotinib, cetuximab, and dasatinib (targeting SRC, EGFR, and other RTKs; ref. 14); minimally sensitized cells to LY294022 (targeting phosphoinositide-3 kinase); and did not sensitize cells to enzastaurin (targeting protein kinase C), MCTP110 (targeting RAS/RAF interactions; ref. 15), rapamycin (targeting mTOR), U0126 (targeting MAP–ERK kinase 1/2), or CPT11 (a DNA-damaging agent). These findings support the selectivity for EGFR in cancer cell lines with activated EGFR signaling (Fig. 1F).

Congruence between Sterol Metabolite Profile and Sensitization to EGFR-Targeting Drugs

We next determined whether production of specific sterol metabolites correlated with sensitization to EGFR inhibitors and was sufficient to explain the observed sensitization. siRNA targeting SC4MOL (sSC4MOL) elevated expression of the SC4MOL substrates 4-mono- and 4,4-dimethylzymosterol (T-MAS; Fig. 2A and Supplementary Fig. S3A), and reduced downstream enzymatic products such as lanosterol. As a contrasting control, depletion of the upstream enzyme, CYP51A1 (Fig. 2A, second row), specifically increased its substrate, dihydrolanosterol. Studies in yeast have previously shown that either chemical or genetic inhibition of the CYP51A1 ortholog, ERG11, rescues lethal mutants in the SC4MOL ortholog, ERG25 (16). Here, the CYP51A1 inhibitor ketoconazole reversed the accumulation of C4-methylsterol substrates in SC4MOL-silenced cells (Fig. 2B) and eliminated the SC4MOL-dependent sensitization of A431 cells to erlotinib (Fig. 2C). Similar results were obtained using siRNA to deplete CYP51A1 (Fig. 2D). As a control, we confirmed that siRNA-depleted SC4MOL levels remained low in ketocanozole-treated cells (Supplementary Fig. S3B), excluding indirect action. Surprisingly, although NSDHL was efficiently depleted by siRNA (Supplementary Fig. S3B), this did not produce accumulation of 4-methylsterols, substrates of functional SC4MOL. This finding may be due to the detection limit of the gas chromatography–mass spectroscopy (GC-MS) technique in identifying low levels of carbonylated derivatives of T-MAS or the enhanced ability of tumor cells to dispose of this metabolite.

Metabolite profiling also showed a small but appreciable reduction of cholesterol in SC4MOL-silenced cells (Fig. 2A) or NSDHL-deficient Bpa1H fibroblasts (Supplementary Fig. S3C). However, supplementation of media with cholesterol or an upstream metabolite in the pathway, such as lanosterol, did not have any effect on viability or sensitivity to EGFR inhibitors (Supplementary Fig. S3D–S3G), suggesting specific effects at the level of the C4-demethylylation complex. In contrast, addition of T-MAS or, most notably, its immediate precursor, folicular liquid meiosis-activating sterol (FF-MAS), to the culture medium reduced cancer cell viability (Fig. 2E) and increased cancer cell sensitivity to erlotinib (Fig. 2F and G). Taken in sum, these data support the interpretation that sensitization to erlotinib is associated with perturbation of pools of a sterol intermediate metabolite proximally upstream of SC4MOL in the metabolic pathway. The negative effect of accumulation of this substrate can be rescued by an upstream blockade, whereas gross changes in the abundance of more distal upstream or downstream sterols (lanosterol and cholesterol) per se are not sufficient to explain the observed effects on EGFR.

Network Modeling Suggests a Role for SC4MOL and NSDHL in Trafficking of EGFR

No previous studies have suggested a mechanism for how the SC4MOL protein might influence sensitization to EGFR inhibitors. Among all sterol-metabolizing enzymes and their corresponding substrates, ERG1, ERG7, ERG11, ERG24, ERG25, ERG26, and ERG27 were conserved between Saccharomyces cerevisiae and humans, such that proteins with high levels of sequence homology carried out comparable functions in sterol biosynthesis (Fig. 1, Supplementary Fig. S4A and S4B, and Supplementary Table S1). The majority of ERG genes downstream of zymosterol (ERG6, ERG2, ERG3, ERG5, and ERG4) showed little or no sequence homology with human genes (KEGG pathways; ref. 17), but, instead, proteins with unrelated sequence carried out comparable enzymatic activities. As a source of insight, we systematically analyzed the yeast orthologs in this highly conserved metabolic pathway. For this, we used the yeast sterol pathway proteins as seeds to mine data from large-scale yeast genetic arrays (18), affinity purification and MS resolution of protein complexes (19–21), and protein complementation screens (22) to gain further insight into their function (Supplementary Fig. S4, Supplementary Table S1, and Supplementary Cytoscape file).

The in silico network generated for ERG25, ERG26, ERG27, and ERG28 proteins (Fig. 3A and Supplementary Fig. S4) revealed, as expected, many interactions reflecting their participation in the linear ergosterol biosynthesis pathway (green circles in Fig. 3A) as well as additional interactions with genes annotated for roles in lipid synthesis and metabolism. Unexpectedly, multiple genetic and protein–protein interactions (PPI) were also detected between ERG25 and proteins with gene ontology annotations, indicating direct involvement in vesicular transport, secretory pathway, and cellular localization: Of 178 ERG25-interacting genes, 53 had such gene ontology annotations, representing a highly significant enrichment (e.g., vesicle-mediated transport, P = 1.4 × 10^{-6}; Fig. 3B, ERG11, which rescues ERG25 mutations, also had many interactions and a significant enrichment for such gene ontology annotations. In contrast, ERG27 and ERG28, which did not affect response to EGFR-targeting agents, interacted with only 8 and 7 nonsterol pathway genes, respectively, and fewer genes overall (Supplementary Fig. S4B). ERG26 had an intermediate number of interactors (n = 46) and no significant gene ontology enrichment. However, genetic and biochemical studies in yeast (12) have noted a close physical and functional interaction between ERG25 and ERG26, suggesting NSDHL might be acting through SC4MOL to influence transport processes. Resistance to cetuximab in the clinic has been strongly linked to defects in internalization and degradation of EGFR (5); we therefore tested the idea that SC4MOL and associated proteins may regulate EGFR trafficking.

Depletion of SC4MOL or NSDHL Increases Vesicular Trafficking of EGFR to the Lysosome

Following activation by EGF binding, or binding by antibodies such as cetuximab, EGFR is internalized from the plasma membrane, moves through a series of sorting endosomes, and then either recycles to the cell surface or moves through the late endosome/multivesicular body (MVB) to...
Figure 2. Concordant effects of siRNA silencing on the cellular composition of sterols. A, sterol composition of cellular pellets was assessed by GC-MS following extraction of lipids. The area under the peak of each sterol species was expressed as the proportion of total cellular sterols, or as per milligram of total protein (for total cholesterol and total sterols). B, ketoconazole treatment for 48 hours prevented accumulation of C4-methylsterols in A431 cells made deficient in SC4MOL with siRNA. In samples parallel to C, C4-methylated sterols were GC-MS measured in total lipid extracts of GL2- and SC4MOL (SC4)–silenced A431 cells. C, effects of the CYP51A1 inhibitor ketoconazole (6 μg/mL) on apoptosis. A431 cells were made SC4MOL deficient by siRNA transfection and, in 24 hours, treated with vehicle or erlotinib for 72 hours, followed by Annexin V surface labeling. Similar results were obtained with cotransfection with 15 nmol/L CYP51A1 siRNA (D). In all samples, the total concentration of siRNA was maintained at 30 nmol/L. P(1), 0.001; (2), 0.001. E, effects of FF-MAS on viability of cancer cell lines. F and G, C4-methylated sterol metabolites, FF-MAS (F), and T-MAS (G), sensitize cancer cell lines to erlotinib. Columns represent averaged results of 3 independent experiments; bars, SDs. Numbers above columns indicate relative increase in erlotinib cytotoxicity, the SI.
Sterol Pathway Genes Regulate Tumor Response to Anti-EGFR

**Figure 3.** Interactions of the ERG25-ERG28 genes in Saccharomyces cerevisiae. **A,** sterol pathway genes interact genetically (blue lines) or physically in protein complexes (red lines), with multiple genes regulating cellular localization (yellow) and vesicular transport (blue) of macromolecules. **B,** GO function Id classification of genes interacting with ERG25–ERG28: ergosterol biosynthesis (6696), secretory pathway (45045), vesicle-mediated transport (16192), localization (51179), membrane lipid biosynthesis (46467), sphingolipid biosynthesis (30148), and ceramide metabolism (6672). The y-axis represents negative logarithm of P value, indicating significance for enrichment considering the entire yeast genome.

the lysosome for destruction (Fig. 4A; ref. 23). After first excluding an effect of SC4MOL deficiency on the rates of EGFR synthesis (Supplementary Fig. S5A), we then systematically probed EGFR trafficking after siRNA depletion of SC4MOL. Levels of cell-surface EGFR declined at a similar rate following EGF stimulation (Fig. 4B), and iodine-125–EGF internalization kinetics were comparable in control or SC4MOL-depleted cells (Supplementary Fig. S5B). We also did not observe differences in the transit of labeled EGF through the early endosomal compartment, as quantified (24) based on colocalization with early endosomal antigen 1 (EEA1; Fig. 4C and Supplementary Fig. S6A).

In contrast, depletion of either SC4MOL or NSDHL significantly reduced the amount of fluorescently labeled EGFR (Fig. 4D) or EGF (Fig. 4E and Supplementary Figs. S5C and S6B) associated with RAB11-positive recycling endosomes after 30 minutes of EGF stimulation. Conversely, SC4MOL- and NSDHL-deficient cells showed an accelerated concentration of internalized fluorescently labeled EGF in RAB7-positive (10 minutes; Fig. 4F and Supplementary Fig. S6C) or EGFR in...
lyosomal-associated membrane protein 1 (LAMP1)-positive late endosomes (Supplementary Fig. S6D), followed by marked loss of EGF in these compartments at later time points (Fig. 4G and Supplementary Fig. S6D and S6E).

Indeed, although basal levels of surface EGFR were comparable, depletion of SC4MOL dramatically reduced total cellular EGFR levels within an hour of EGF treatment (Fig. 4H and I), with more than 80% loss of EGFR within 4 hours of EGF treatment. Interestingly, ERBB2 and ERBB3, capable of binding membrane EGFR (but not EGF), also underwent similar rapid elimination in SC4MOL-targeted cells following EGF-induced EGFR internalization to endosomes (Fig. 4I), whereas an unrelated surface protein, E-cadherin, did not (Fig. 4I). Similar findings were observed with direct labeling of EGFR in SC4MOL-deficient cells treated with EGF for 30 and 60 minutes, which showed substantial depletion of EGFR in SC4MOL-deficient cells (Supplementary Fig. S6D). Ubiquitin-conjugated immunoprecipitated forms of EGFR accumulated with accelerated kinetics in SC4MOL- or NSDHL-deficient cells, compatible with early arrival at the late endosomes (Fig. 4K and Supplementary Fig. S7A–S7C). Finally, treatment with the antimalarial primaquine, which inhibits lysosomal acidification, or the CYP51A1 inhibitor ketoconazole, rescued EGFR levels in SC4MOL- and NSDHL-depleted cells, whereas the proteasomal inhibitor bortezomib did not (Supplementary Fig. S7D and S7E). In keeping with the possibility of rapid lysosomal clearance of EGF–EGFR complexes in SC4MOL-depleted cells, the Cys/Ser/Thr peptidase inhibitor leupeptin (25) also eliminated loss of fluorescently labeled EGF (compared with GL2 and CYP51A1 in Fig. 4G and Supplementary Fig. S6E).

Depletion of SC4MOL Suppresses EGFR-Dependent Signaling, Causes EGFR Loss, and Induces In Vivo Sensitization to Cetuximab

 Trafficking of EGFR to late endosomes and lysosomes has been shown to rapidly terminate its signaling (26). We investigated EGFR signaling in cells with depleted SC4MOL, on the basis of the observation of aberrant EGFR trafficking in these cells (Fig. 4). Indeed, SC4MOL siRNA-treated SCC61 cells pulsed with EGF showed reduced total and phosphorylated EGFR as well as reduced phosphorylation of its key downstream effectors, extracellular signal–regulated kinase (ERK), AKT (phospho-S473), S6K, 4EBP1, and S6 ribosomal protein (Fig. 5A and Supplementary Fig. S8A).

We used shRNA to deplete SC4MOL in A431 cells, and obtained 2 independent lines for xenograft analysis and a nontargeting control–depleted line (Supplementary Fig. S2B). Lysates of xenografted A431 tumors obtained 72 hours after a single intraarterial dose of cetuximab delivered on day 8 after implantation showed reduction of total EGFR, and marked suppression of phosphorylation of EGFR, its heterodimerizing partner ERBB2, and the downstream signaling effectors AKT, S6K, S6, and ERK (Fig. 5B and C) in SC4MOL-deficient versus control xenografts. If depletion of SC4MOL sensitizes cells to EGFR inhibitors by promoting EGFR degradation, it should be particularly effective in sensitizing tumors to cetuximab in vivo, given the action of this antibody in promoting EGFR lysosomal degradation (27). Growth of SC4MOL-depleted tumors was moderately suppressed compared with control-depleted A431 cells (P = 2.2 × 10−16, Fig. 5D). Cetuximab treatment of control-depleted tumors beginning at day 8 after injection of mice with tumors transiently reduced tumor growth, which resumed after approximately 2 weeks (Fig. 5D). In striking contrast, similar cetuximab treatment of SC4MOL-depleted tumors almost completely prevented growth of SC4MOL-deficient xenografts (Fig. 5D), and 2 to 3 of 10 animals in each shRNA group showed no evidence of tumor at 7 weeks. Similar to the conserved ERG11/CYP51A1 and ERG25/SC4MOL epistasis (16) in vitro (Fig. 2B–D), SC4MOL-deficient A431 xenografts resisted cetuximab in mice given fluconazole, a CYP51A1 inhibitor, in the drinking water (Supplementary Fig. S8B). The effect of SC4MOL silencing on EGFR signaling was further evaluated in the context of the EGFR kinase inhibitor erlotinib (Fig. 5E) in cells treated with EGF to induce receptor internalization (28). The SC4MOL-depleted cells showed higher sensitivity to erlotinib-induced suppression of AKT, S6 kinase, and S6 phosphorylation, whereas levels of p-Y1173-EGFR and p-ERK were equally suppressed by erlotinib in SC4MOL-deficient cells and controls (Fig. 5E).

Network-Predicted SC4MOL Interactions Control EGFR Endocytic Traffic

Given the clear success of the yeast protein interaction network in suggesting a role for SC4MOL and NSDHL in EGFR protein trafficking (Fig. 3A and Supplementary Fig. S4), we hypothesized that these data could also nominate SC4MOL effectors relevant to this process, given the growing appreciation of the existence of interologs (evolutionarily conserved interactions) among highly conserved proteins and protein modules (29). We therefore identified human orthologs for those yeast proteins shown in Fig. 3A that possess gene ontology annotations relevant to vesicular trafficking (Fig. 6A and Supplementary Table S1). Our analysis predicted that ERG25/SC4MOL and ERG26/NSDHL might interact directly with multiple components of the mammalian exocytic machinery, including COPI, the p24 cargo receptors TMED2 and TMED10, or the ADP ribosylation factor (ARF) GTPases (18, 30, 31). This prediction was particularly interesting, as we had identified ARF4 and ARF5 as hits in the initial screen yielding SC4MOL as a sensitizer to EGFR-targeting drugs (7), and as these proteins are known to form a complex that regulates the trafficking of EGFR out of the Golgi and late endosomes via a RAB11-mediated recycling pathway (32).

Systematically probing the ARF subnetwork, we found that siRNA depletion of several components of this network increased sensitivity of the A431 cancer cell line to EGFR inhibition (Fig. 6B; ref. 7). Furthermore, silencing of ARF4 and ARF5 increased accumulation of labeled EGF in RAB7-positive endosomes and reduced entry of EGF into RAB11 compartments (Fig. 6C–E), paralleling the SC4MOL depletion phenotype. Although ketoconazole, an inhibitor of CYP51A1, rescued the effects of SC4MOL depletion (i.e., through elimination of metabolite accumulation), it did not reverse the EGFR trafficking defects due to ARF4 and ARF5 silencing, in keeping with our model in Fig. 6A. We also found that EGFR and ARF5 colocalized in the leading cellular contact-free edge of control-silenced A431 cells (Fig. 6F, siGL2 and siCYP51A1), consistent with the critical role of ARF4 and ARF5 in trans-Golgi trafficking and exocytosis (33). This colocalization was
Sterol Pathway Genes Regulate Tumor Response to Anti-EGFR

Figure 4. Altered endosomal trafficking of EGFR in SC4MOL- and NSDHL-deficient cells. A, simplified schema of EGFR endocytosis and markers of compartments. B, kinetics of EGFR internalization in siRNA-depleted cells as measured by surface EGFR. C, colocalization of labeled EGFR with Rab11-positive recycling endosomes (note depletion of EGFR at 60 minutes). D, EGF loss at 1 hour in SC4MOL-silenced cells was prevented by leupeptin or CYP51A1 silencing.

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Figure 5. Silencing of SC4MOL suppresses EGFR signaling and sensitizes A431 xenografts to cetuximab. **A**, silencing SC4MOL in SCC61 cells suppresses phosphorylation of EGFR downstream effectors following EGF stimulation for indicated time intervals. **B**, effects of a single dose of cetuximab on A431 xenografts at 72 hours. Numbers below bands are tubulin-normalized densities relative to vehicle-treated shControl grafts. **C**, summary of results quantified from **B**, *P* < 0.05. Black bars represent cetuximab (Cet), and gray bars represent vehicle (V). **D**, tumor volumes of xenografts of shRNA-modified A431 cells treated with cetuximab (closed symbols) or vehicle (open symbols); *P*(1) = 2.2 × 10^{-16}. **E**, SCC61 cells depleted of SC4MOL were sensitized to erlotinib-induced suppression of AKT–mTOR pathway signaling. Graphs represent averaged results of 3 experiments; bars, SEM.
completely blocked by silencing of SC4MOL or NSDHL (Fig. 6F). These data are compatible with the idea of an important contribution of SC4MOL and NSDHL to EGFR endocytic traffic within ARF4- and ARF5-positive endosomes.

**Genetic Loss of Nsdhl Reduces EGFR Expression and Signaling In Vivo**

Finally, to determine whether our data offer new insights into the physiologic control of EGFR activity in vivo, we took advantage of the bare patches (Bpa+/−) mouse model, which contains a nonsense mutation (K103X) in the X-linked Nsdhl gene (34). This mutation results in a null allele with a truncated protein that is degraded. This genetic lesion in heterozygous Bpa+/− females produces mosaic NSDHL-null regions of the skin with characteristic patchy, scaly eruptions by postnatal day 5 that subsequently resolve, leaving linear stripes following lines of X-inactivation. Strikingly, these NSDHL-null areas of the skin (between the dashed lines in Supplementary Fig. S9A) differed from the wild-type areas in the patterns of EGFR expression, activation, and signaling. Total EGFR staining (Fig. 7A and Supplementary Fig. S9B and S9C) was predominantly membranous in the NSDHL-null areas. The nuclear EGFR was significantly reduced in the mutated patches of the skin (Fig. 7A and Supplementary Fig. S9C). The reduced intracellular EGFR staining was accompanied by a marked loss of phosphorylated (activated) Y173-EGFR (Fig. 7A and Supplementary Fig. S9D), T202/Y204-ERK1/2 (Fig. 7A and Supplementary Fig. S9E), and S473-AKT (Fig. 7A and Supplementary Fig. S9F) signal in the NSDHL-null areas. EGFR signaling is of critical importance for keratinocyte proliferation and differentiation (35). The NSDHL-null areas of the skin also exhibited significantly reduced proliferation, as determined by Ki-67 (Fig. 7A and Supplementary Fig. S9G). The reduced expression of NSDHL protein in the proliferating basal layer (Fig. 7A and Supplementary Fig. S9G) expression and in vivo bromodeoxyuridine (BrdUrd) incorporation (Supplementary Fig. S9H). Global effects of loss of NSDHL protein expression were not observed, as expression of keratin-14 was normal (Fig. 7A and Supplementary Fig. S9I). However, the Nsdhl-mutated areas consistently showed parakeratosis accompanied by reduced expression of loricrin, a marker of terminally differentiated keratinocytes (Supplementary Fig. S9J).

Using NSDHL-deficient mouse embryonic fibroblasts derived from these mice, we next assessed whether a ligand-induced trafficking defect was observed for an RTK commonly expressed in fibroblasts, platelet-derived growth factor receptor (PDGFR). Semiquantitative analysis of PDGFR levels in these fibroblasts treated with PDGF showed accelerated ligand-induced PDGFR loss in NSDHL-deficient Bpa+/− cells, but not in controls; the former was associated with concentration of PDGFR in RAB7 endosomes (Fig. 7B and C).

Finally, although no suitable immunohistochemistry antibody reagents were available to analyze SC4MOL, increased expression of NSDHL protein in the proliferating basal layer of normal oral keratinocytes (Supplementary Fig. S10A), was observed, thus suggesting a critical requirement of the sterol pathway enzymes for EGFR-dependent cellular growth. We further conducted quantitative measurements of NSDHL, using automated quantitative analysis (AQUA; ref. 36), and found NSDHL expression was more abundant in squamous cell head and neck carcinomas compared with normal epithelium (Fig. 7D and Supplementary Fig. S10B and S10C), but did not affect the survival of squamous head and neck carcinoma patients (Supplementary Fig. S10D).

**DISCUSSION**

We here use a network modeling–guided strategy to show that depletion of SC4MOL and NSDHL, best known as components of a C4-demethylase complex in the distal cholesterol pathway, potently sensitizes tumor cells to EGFR inhibitors, or could be directly cytotoxic in cancer cells with low EGFR. Our data suggest that the mechanistic basis for this effect is a requirement for SC4MOL and NSDHL for effective endosomal trafficking of EGFR, thus limiting EGFR stability and the availability of an activated receptor pool. Mechanistically, the activity of SC4MOL and NSDHL mediates the production of specific sterol metabolites and may also involve proteins regulating the endocytic recycling of receptors (such as ARF1, ARF4, and ARF5), as inferred from the yeast interaction data.

Many studies have implicated function of the sterol synthesis pathway in tumor growth and response to treatment. For example, the sterol composition of the membrane has been shown to regulate EGFR signaling (26) and sensitivity of head and neck cancer cells to apoptosis (37). Inhibition of lipogenesis has been an effective antitumor strategy in glioblastoma models associated with hyperactive EGFRvIII (38). We found increased expression of NSDHL in head and carcinoma tissues (Fig. 7D), in agreement with reports on accelerated sterol biosynthesis in cancer, which is associated with drug resistance and poor survival in breast and non–small cell lung cancer (39, 40). However, in contrast to upstream pathway components such as farnesyltransferases, and downstream components directly regulating cholesterol production, SC4MOL and NSDHL have not been previously analyzed in the context of cancer signaling.

Our data imply a particularly important and previously unappreciated functional role for SC4MOL and NSDHL in vesicular trafficking. We observed a significant bias in routing the EGFR-containing endosomes toward RAB7 late endocytic compartments as opposed to RAB11 recycling compartments (Fig. 5 and Supplementary Fig. S6). Such alteration may promote rapid EGFR dephosphorylation via membrane contact with PTP1B (41) or other EGFR phosphatases. Studies of NSDHL have also indicated this protein traffics through the Golgi and then associates with ER membranes and lipid droplets (42). Lipid droplets are ER-derived structures that serve to compartmentalize lipids and an array of enzymes, kinases, and other proteins. Lipid droplets have attracted increasing attention because of the evidence that they mediate trafficking of signaling proteins to multiple organelles, and have roles in cell signaling and secretion during inflammation and cancer (43). A recent proteomic study also noted enrichment of NSDHL at lysosomes during camptothecin-induced apoptosis, implying mobilization of NSDHL-containing vesicles to this structure during induction of cell death (44). Work reported here for the first time suggests that SC4MOL- and NSDHL-dependent trafficking, potentially in association with targeted delivery of specific metabolites, actively contributes to the pathologic features and drug resistance of cancer.

The C4-methylsterols, FF-MAS and T-MAS, are the substrates for the evolutionarily conserved LBR/TM7SF2/Erg24p
Figure 6. Network-predicted SC4MOL interactions control EGFR endocytic traffic. A, network of yeast and human interologs for ERG25/SC4MOL and EGFR-interacting proteins. Interactions inferred from yeast are shown as green dashed lines. Circles are nodes represented by individual proteins or paralogs. Blue lines depict mammalian interactions; thickness is proportionate to the confidence score of an interaction. B, silencing of ERG25/SC4MOL-interacting proteins increased cytotoxicity of erlotinib. C and D, depletion of SC4MOL, and its interacting ARF4 and ARF5 proteins, promoted EGF localization to late RAB7 endosomes (C and representative images in E, arrowheads) and suppressed EGF entry into RAB11-recycling compartments (D). Ketoconazole pretreatment selectively rescued the EGF-trafficking defect in SC4MOL-silenced cells (C, D, closed columns). F, ARF5 and EGFR were expressed at the growing edge of A431 cells (arrows). Silencing of SC4MOL and NSDHL reduced EGFR and ARF5 colocalization. n, nuclei marked with dashed lines; solid lines, cell borders. *, P < 0.001 in all graphs; when 2 asterisks appear next to each other above 2 columns, it means that both vehicle and ketoconazole are different from GL2; scale bars in all images, 10 μm.
Sterol Pathway Genes Regulate Tumor Response to Anti-EGFR

and SC4MOL/Erg25p enzymes, respectively. Strikingly, we discovered new signaling inhibitory properties of these sterol metabolites, which have previously been studied primarily in the context of gonadal physiology (45, 46). FF-MAS and testis meiosis-activating sterol (T-MAS) have significant biologic activities in regulating cumulus expansion and oocyte maturation. Some studies of reproductive biology (47, 48) indicate that this action likely involves regulation of EGFR signaling, which is known to be important during ovulation and cumulus dispersal (49). Our data for the first time show activity for these proteins in resistance to erlotinib in cancer cells: We hypothesize that the addition of large pools of external FF-MAS and T-MAS disrupts the vesicular-targeting role of small endogenous pools of these metabolites.

Although 4-methylcholest-8(9)-en-3β-ol and 4,4′-dimethylcholest-8(9)-en-3β-ol intermediaries were detected in SC4MOL-deficient tumor cells and in Bpa1H fibroblasts with germine nonsense mutation in exon 5 of Nsdhl (34), we did not detect these intermediates following depletion of NSDHL with siRNA (Fig. 2A) in carcinoma cell lines. This discrepancy likely results from the fact that these methylsterses are direct substrates of SC4MOL, not NSDHL, and potentially results from the low residual levels of NSDHL protein due to incomplete knockdown. We thoroughly validated the idea that both SC4MOL and NSDHL regulate trafficking comparably (Fig. 4 and Supplementary Fig. S6). By application of C4-methylsterols in vitro, and by pharmacologic inactivation of the upstream CYP51A1 with ketoconazole or by siRNA knockdown (Fig. 2 and Supplementary Fig. S3), we showed a direct effect of SC4MOL and NSDHL on drug sensitivity. Our data also emphasize interactions between ERG25/SC4MOL and multiple genes associated with endosomal compartments (Fig. 3). Taken in sum, these results support the interpretation that grossly regulating the abundance of a sterol intermediate substrate, or depletion of cholesterol, the end prouct of the pathway, is not sufficient per se to explain the observed sensitization and trafficking effects. One plausible explanation is that the localized insertion of sterol intermediates into specific vesicular compartments facilitates targeting to specific destinations, as in the pathway recently defined in Arabidopsis (50).

Perturbations of sterol metabolism in humans and in animal models cause skin manifestations associated with accumulation of sterol metabolites, but the mechanism was largely unclear. Although no SC4MOL mouse model exists, a patient with SC4MOL deficiency who presented with marked diffuse psoriasiform skin rash has been described (51). Mutations in NSDHL are associated with human CHILD syndrome (congenital hemidysplasia with ichthyosiform nevus and limb defects), an X-linked, male lethal disorder (52). Similar to the human NSDHL deficiency syndrome (53) or the X-linked dominant Conradi–Hunermann–Happle syndrome (54), skin manifestations in the respective Bpa1H and tattered murine models of these sterol pathway disorders include atrophic and pigmentary lesions, striated hyperkeratosis, coarse lusterless hair, and alopecia. These skin changes are similar to those seen in mice and humans with the EGFR pathway perturbations (35). Our studies indicate that these phenotypes may indeed be connected, as EGFR signaling is markedly downregulated in skin keratinocytes with loss of Nsdhl (Fig. 6).

Targeting EGFR in carcinomas has produced tangible therapeutic gains in the treatment of colorectal, breast, head and neck, and non–small cell lung cancers. However, the fact that only a small subset of tumors are controlled by EGFR-directed therapies makes it critical to identify biomarkers for...
patient response, or targets for drugs that can be combined in treatment strategies that boost the efficacy of EGFR inhibitors. In this context, blockade of sterol metabolism at the level of SC4MOL or NSDHL provides a potential unique point to influence resistance to EGFR-targeted inhibitors via regulation of EGFR intracellular traffic and signaling activity. Moreover, the fact that depletion of SC4MOL and NSDHL also targets dimerization partners of EGFR, such as ERBB2 and ERBB3, and potentially other proteins that are commonly upregulated to compensate for resistance to EGFR inhibitors, makes this a particularly attractive strategy, as receptor targeting strategies at the level of EGFR endocytic trafficking have begun to show promising activity (55).

Finally, the approach we develop here illustrates the power of integrating systems biologic analysis of protein networks into efforts to elucidate the function of relatively obscure proteins. Our work has moved from initial generation of an EGFR-focused network to guide siRNA screening based on the hypothesis that proteins linked to EGFR by some criteria—physical, genetic, or expression change—would be more likely to modulate EGFR function (7). Having identified SC4MOL, we then modeled from orthologs and a network of their protein interactions in yeast to generate a new hypothesis regarding protein function. Having validated the functional prediction of a role in vesicular trafficking, we were then able to rapidly model interologs relevant to protein action, identifying relevant human protein partners that closed the circle by establishing a proximal link to EGFR trafficking. The success of this rapid oscillation between hypothesis generation and hypothesis testing illustrates the powerful resources becoming available from “omics” projects, and should support more rapid development of these projects for clinical use in the future.

METHODS

Cell Lines, Compounds, and Antibodies

The A431, FaDu, Detroit, and MCF12F cells were obtained from the American Type Culture Collection and maintained at the Fox Chase Cancer Center Cell Culture Facility (Philadelphia, PA). The identity of the A431 cell line was confirmed by single tandem repeat DNA profiling (Biosynthesis). The SCC61, SCC68, and SCC25 cells were kindly provided by Dr. Tanguy Y. Seiwert (University of Chicago, Chicago, IL). PC9 cells were kindly provided by Dr. William Pao (Vanderbilt-Ingram Cancer Center, Nashville, TN). All cell lines were mycoplasma free and maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% v/v FBS and l-glutamine without antibiotics. Mouse embryonic fibroblasts were obtained from B6/n mice as described (56) and propagated in DMEM supplemented with 10% v/v FBS and l-glutamine without antibiotics. Cetuximab and erlotinib were obtained from the Fox Chase Cancer Center pharmacy; CPT-11, cholesterol, and lanosterol were purchased from Sigma-Aldrich; FF-MAS (folicular fluid meiosis-activating sterol) (14-dimethyl-14-dehydrolanosterol, cat. no. 700077) and T-MAS (testicular meiosis-activating sterol) (4,4-dimethylzymosterol/4,4-dimethyl-14-dehydrolanosterol, cat. no. 700073) were purchased from Avanti Polar Lipids, Inc. All antibodies used in Western blot experiments were purchased from Cell Signaling. Rabbit polyclonal antibody to SC4MOL was raised using the peptide antigen DEAVERTVQSFHHLRKDK (amino acid residues 345–362) of mouse NSDHL.

siRNA Transfections and In Vitro Viability Assays

The siRNA targeting human sterol biosynthesis genes and controls were obtained from Qiagen. Cells were transfected in triplicates with siRNA at 10 nmol/L concentrations mixed with HiPerFect Transfection Reagent (Qiagen) on a 96-well plate according to the manufacturer’s reverse transfection protocol. Twenty-four hours after plating, cells were treated with erlotinib, cetuximab, CPT-11, or vehicle [0.02% dimethyl sulfoxide (DMSO)]. The viability was measured in 96 hours using CellTiter-Blue Viability Assay (Promega). We used a sensitization index (SI) to determine if gene silencing enhances drug cytotoxicity (7). Viability was determined for each target gene and normalized to the averaged GL2 viability on each plate. SI was calculated for each individual well as SI = (XsiRNA/GL2siRNA)/(XDMEM/GL2DMEM), where X was viability in wells transfected with targeting duplexes and GL2 was the averaged viability of 3 wells with nontargeting negative control siRNA on the same plate. The SI = 1 indicates no interaction, and SI < 1 indicates potentiation of the drug cytotoxicity. In viability assays with sterol metabolites, the latter were added with the drugs. Corresponding dilutions of ethanol and DMSO were added as vehicle and did not affect the viability, compared with that of untreated cells.

Quantitative RT-PCR

For evaluation of target gene knockdown, cells were reverse transfected in 6-well plates, and total RNA was extracted using the RNeasy Mini Kit (Qiagen) 48 hours after transfection. Quantitative reverse-transcriptase PCR reactions were conducted using TaqMan probes and primers designed by the manufacturer, using an ABI PRISM 7700 Detection System (Applied Biosystems). The results were analyzed with the comparative CΔ method to establish relative expression curves.

Apoptosis and Pathway Analysis

Apoptosis was measured using the Annexin V assay (Guava Technologies). Annexin V-positive A431 cells were counted using Guava flow cytometry 72 hours after transfection, 48 hours after treatment. To measure the effect of siRNAs on the activity of EGFR effectors, cells were transfected with siRNA, and the culture media replaced with glutamine-supplemented serum-free DMEM 24 hours after transfection. After overnight incubation, cells were either left untreated or stimulated with EGFR at 15 ng/mL for 15 minutes. Cell extracts were prepared using M-PER Mammalian Protein Extraction buffer (Thermo Scientific) supplemented with the Halt Phosphatase Inhibitor Cocktail (Thermo Scientific) and the Complete Mini Protease Inhibitor Cocktail (Roche Diagnostics GmbH). Extracts were centrifuged at 15,000 × g for 10 minutes at 4°C. Western blot analysis was conducted using antibodies to phosphorylated and total EGFR, AKT and ERK1/2 and to β-actin (Cell Signaling).

Analysis of Sterol Composition

Sterol contents of A431 cells cultured in 1% FBS/DMEM were measured using selected ion monitoring GC-MS as described by Kelley (57). Briefly, cells were transfected with 30 nmol/L siRNA and, in 72 hours, detached from the plastic by trypsin. Two aliquots per sample were stored at −80°C until analysis for total protein and lipids.

Immunofluorescent Microscopy

A431 and SCC61 cells were seeded on glass slides in the presence of 30 nmol/L siRNA. For EGFR internalization studies, cells were starved overnight in serum-free DMEM/l-glutamine and, at 72 hours after transfection, were labeled on ice (basal) with 100 ng/mL of EGF Alexa Fluor 488 for 1 hour as previously described (24). Then, the medium was changed to prewarmed DMEM supplemented with l-glutamine and transferred to a 37°C incubator for 10 or 30 minutes and fixed with freshly prepared 4% paraformaldehyde (Electron Microscopy Sciences). For later time points, the internalization proceeded for 30 minutes, after which cells
Sterol Pathway Genes Regulate Tumor Response to Anti-EGFR

were washed twice in DMEM/glutamine and chased for up to 3 hours in the presence of unlabeled EGF at 100 ng/mL to avoid binding of labeled EGF recycled to the cell surface. After 2 washes in ice-cold PBS, cells were fixed in freshly prepared 4% paraformaldehyde/PBS at ambient temperature for 10 minutes, followed by 2 washes in PBS and stored at 4°C or labeled immediately. In some EGF internalization experiments, cells were pretreated with leupeptin at 21 μmol/L at 37°C for 1 hour. In these experiments, the same concentrations of inhibitors were maintained during incubations with EGF (25).

For RAB7 (Cell Signaling) and RAB11 (Invitrogen) antibody immunolabeling, cells were incubated in PBS with 5% normal bovine serum and 0.3% Triton X-100, followed by overnight incubation with primary antibodies diluted 1:100 in 1% BSA/0.3% Triton X-100/PBS and appropriate secondary antibody conjugated with Alexa Fluor 488 and Alexa Fluor 568 (Invitrogen). For experiments with ARF1, ARF4, ARF5, LAMP1, and EE1 (Abcam), all incubations were conducted in the presence of 0.05% Triton X-100. All immunofluorescent imaging experiments were repeated at least twice. In multiple spots (4–5) of each slide, 10 μL of mixture of antibodies diluted 1:500 in 1% BSA/0.3% Triton X-100/PBS were applied, and the slides were covered with coverslip and were incubated for 1 hour at room temperature and then washed three times in PBS. Coverslips were removed using a small difficulties.

Mouse Skin Tissue Collection and Immunohistochemistry

Following sacrifice at postnatal day 3, skin over the dorsum of the back was removed and processed for immunohistochemical staining. Skin was fixed in Bouin’s fixative and stained with NSDHL antibody overnight at 4°C. Heat was used for antigen retrieval (95°C for 20 minutes) in solution (10 mmol/L sodium citrate, 0.05% Tween 20, pH 6). Slides were incubated with primary antibodies (Cell Signaling) diluted per the manufacturer’s instructions. Primary antibody binding was visualized using the Liquid DAB+ Substrate Chromogen System (Dako). Samples were counterstained with hematoxylin.

Network Assembly and Analysis

Publicly available databases [BioGrid (58), SBD (59), and STRING (60)] were searched for the yeast ergosterol biosynthesis pathway genes, which were used as search seeds (Supplementary Fig S4). Orthologs for yeast genes were identified in higher eukaryotes using PubMed and P-POD (Princeton Protein Orthology Database; ref. 61, using Naive Ensemble search tool). Subsequent analysis was aimed to augment information on PPIs in human cells, PPIs between homologous genes in model organisms, database or pathway links, and text mining (coappearance of gene names in PubMed). Data regarding experimentally proven interactions in human and model organisms were merged in Cytoscape (Supplementary File and Supplementary Table S1). To provide additional context in some analyses (Fig. 4), STRING-extracted information from pathway databases and text-mining data.
were merged and displayed using Cytoscape as indicated in the figure legends. BINGO plugin to the Cytoscape was used to identify gene ontology functions overrepresented in the ERG25–ERG28 interactions network in Fig. 3; the nodes were color labeled using GOlorize plugin to the Cytoscape.

**Statistical Analysis**

For drug sensitivity testing and Western blot enumeration of protein expression, we used generalized linear models assuming gamma family and log link. For analysis of immunofluorescent imaging, the Student 2-sided t test was used after visual assessment for normality of data distribution.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Acknowledgments

The authors thank Calvin Shaller for technical assistance with Western blots, Fox Chase Genomics Facility (Dr. Emmanuelille Nicolas), and Dr. Jonathan Chernoff for critical comments on the work.

**Grant Support**

This work was supported by NIH core grant CA-06927, by the Pew Charitable Fund, and by a generous gift from Mrs. Concetta Greenberg to Fox Chase Cancer Center. Some of the authors were supported by NIH R01 CA-63366 and SPORE P50 CAD83638 (to E.A. Golemis); and by Tobacco Settlement funding from the State of Pennsylvania (to E.A. Golemis); and by NIH R01 CA50633, U54 CA141947, and by the Jeanne Littledfield Award from the American Association of Cancer Research (to L.M. Weiner); by NIH R01 HD38572 (to G.E. Herman); and by NIH K22 CA160725, R21 CA164205, and a career development award from Genentech (to I. Astsaturov).

Received January 25, 2012; revised September 26, 2012; accepted October 24, 2012; published OnlineFirst November 2, 2012.

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Sterol Pathway Genes Regulate Tumor Response to Anti-EGFR

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Published OnlineFirst November 2, 2012; DOI: 10.1158/2159-8290.CD-12-0031
Targeting C4-Demethylating Genes in the Cholesterol Pathway Sensitizes Cancer Cells to EGF Receptor Inhibitors via Increased EGF Receptor Degradation

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Cancer Discovery  Published OnlineFirst November 2, 2012.

Updated version  Access the most recent version of this article at: doi:10.1158/2159-8290.CD-12-0031

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