Dsh Homolog DVL3 Mediates Resistance to IGFIR Inhibition by Regulating IGF-RAS Signaling

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Abstract

Drugs that inhibit insulin-like growth factor 1 (IGF1) receptor IGFIR were encouraging in early trials, but predictive biomarkers were lacking and the drugs provided insufficient benefit in unselected patients. In this study, we used genetic screening and downstream validation to identify the WNT pathway element DVL3 as a mediator of resistance to IGFIR inhibition. Sensitivity to IGFIR inhibition was enhanced specifically in vitro by genetic or pharmacologic blockade of DVL3. In breast and prostate cancer cells, sensitization tracked with enhanced MEK–ERK activation and relied upon MEK activity and DVL3 expression. Mechanistic investigations showed that DVL3 is present in an adaptor complex that links IGFIR to RAS, which includes Shc, growth factor receptor–bound-2 (Grb2), son-of-sevenless (SOS), and the tumor suppressor DAB2. Dual DVL and DAB2 blockade synergized in activating ERKs and sensitizing cells to IGFIR inhibition, suggesting a nonredundant role for DVL3 in the Shc–Grb2–SOS complex. Clinically, tumors that responded to IGFIR inhibition contained relatively lower levels of DVL3 protein than resistant tumors, and DVL3 levels in tumors correlated inversely with progression-free survival in patients treated with IGFIR antibodies. Because IGFIR does not contain activating mutations analogous to EGFR variants associated with response to EGFR inhibitors, we suggest that IGF signaling achieves an equivalent integration at the postreceptor level through adaptor protein complexes, influencing cellular dependence on the IGF axis and identifying a patient population with potential to benefit from IGFIR inhibition.

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Introduction

Type 1 insulin-like growth factor 1 receptor (IGFIR) signals via recruitment of adaptors, including insulin receptor substrate-1 (IRS-1) and Shc, to drive proliferation, invasion, and cell survival (1). In the clinical and experimental setting, low IGF bioactivity protects from tumor development and metastasis, suggesting that IGFs provide a potent protumorigenic signal (2, 3). In early clinical trials, IGFIR inhibitory drugs induced objective regressions, some dramatic and durable, as monotherapy in Ewing sarcomas and other uncommon tumors, and with chemotherapy or targeted agents in common cancers (4–9). However, these encouraging early reports have not translated to phase III benefit in unselected patients (10). Clearly, a better understanding of IGFIR biology is required if this therapeutic approach is to be successful. Key priorities are to understand what makes tumors resistant to IGFIR inhibition, use this information to target these drugs to potentially responsive patients, and select rational treatment combinations for clinical testing. To address these issues, we used genetic screening and downstream validation to identify novel determinants of resistance to an IGFIR antagonist.
Materials and Methods

Cell lines and reagents
DU145, PC3, LNCaP, and LNCaP-LN3 prostate cancer and MCF-7, MDA-MB-231, and MDA-MB-468 breast cancer cells were from Cancer Research UK Cell Services (Clare Hall Laboratories), 22Rv1 prostate cancer cells from the ATCC, and BT20 and BT549 from Dr. Anthony Kong, University of Oxford, Oxford, United Kingdom. All cell lines were mycoplasma-free when tested with MycoAlert (Lonza Rockland Inc.). AZ12253801 and gefitinib were provided by AstraZeneca. DVL-PDZ inhibitor II (DVLi) purchased from Calbiochem, and BMS-754807, XAV939, and AZD6244 from Selleck Chemicals. DVL3 cDNA (Addgene) was amplified using primers 5′-GGATCCATGGACTACAAGGACGACGACGA-3′ and 5′-CTCGAGTCACATCACATCCACAAA-GAAC-3′ incorporating BamH1 and Xho1 sites, respectively (underlined), digested with BamH1 and Xho1 (New England Biolabs), cloned into BamH1-Xho1−digested pHRSIN-CSGW HIV vector, and after verification of the insert by DNA sequencing, used for virus production as described (11). Western blotting, immunoprecipitation, and pulldown assays were performed using reagents described in ref. 12, Supplementary Methods, and Supplementary Table S1.

siRNA screens
Screens were performed as described previously (13 and 14), using kinase siRNA library (siARRAY, targeting 779 known and putative human protein kinases; Dharmacon) containing SMARTPools of four siRNAs targeting each transcript, and human DNA Repair siRNA Set V1.0 siRNA library (Qiagen), together with siPLK1 and Allstars siRNA as positive and negative controls, respectively. Cells were reverse-transfected with 50 nmol/L siRNAs using Dharmafect 1 reagent for DU145 and Dharmafect 3 for MCF-7. Two days later, cells were exposed to vehicle (0.01% DMSO) or AZ12253801 at the GI50 for 5 days, and viability was assessed by CellTititer Glo (CTG) assay (Promega). Duplicate primary screens were analyzed to derive Z′-factors of 0.23 to 0.6 (Supplementary Table S4) indicating good discrimination between positive and negative controls (15). We calculated drug sensitization Z-scores (13) for each siRNA, rank-ordered siRNAs by Z-score, and selected 54 genes for validation (Supplementary Table S5). Triplet second-round screens identified 12 putative resistance mediators, including regulators of the cell cycle and DNA damage response, and proteins with poorly characterized functions (Fig. 1A). Seven hits in DU145 screens (CDKN2C, CNKSR1, DUSP5, HUNK, LMTK3, MPP2, and DVL3) were also candidate hits in MCF7 cells. Validation in low-throughput format confirmed that depletion of each hit enhanced AZ12253801 sensitivity (Supplementary Table S6).

DVL3 mediates resistance to IGFIR inhibition
Known cross-talk between the IGF and WNT axes (19–21) prompted us to investigate one of the most robust hits: Dishevelled homolog 3 (DVL3), a poorly characterized WNT component. DVL3 is one of three mammalian homologs of Drosophila Dsh, a cytoplasmic protein that is phosphorylated on binding of Wnts to Frizzled (Fz) receptors, blocking the β-catenin destruction complex (22). DVL3 was the dominant isoform in both DU145 and MCF7 cells (Fig. 1B), and depletion of DVL3 but not DVL 1 or 2 sensitized to AZ12253801 (Fig. 1C and D). Expression of siRNA-resistant FLAG-DVL3 was able to rescue from AZ12253801 sensitization induced by DVL3 siRNA targeting the 3′-UTR of endogenous DVL3 mRNA (Fig. 1E), suggesting that sensitization by DVL3 depletion was unlikely to be an off-target effect of DVL3 siRNA. As noted above, AZ12253801 can also inhibit the closely related INSR. Although
DVL3-depleted DU145 and MCF7 cells were sensitized to IGFIR depletion, INSR-depleted cells showed no significant reduction in viability upon DVL3 depletion (Fig. 1F and Supplementary Fig. S1C), supporting the contention that functional interaction between DVL3 and AZ12253801 is related to the ability of AZ12253801 to block IGFIR. We next tested Figure 2. DVL3 depletion enhances IGF signaling via MEK–ERK. A, DU145 cells were Allstars (AS) or DVL3 siRNA–transfected, and after 48 hours, treated with 10 nmol/L IGF1 for 10 minutes. Right, graphs, mean ± SEM phospho-IGFIR, phospho-AKT, phospho-MEK, and phospho-ERKs corrected for equivalent total proteins, expressed as % IGF-treated Allstars-transfectants from three independent experiments (*, P < 0.05; **, P < 0.01; ***, P < 0.001, by one-way ANOVA). B, Allstars or DVL3 siRNA–transfected cells were serum-starved for 12 hours and stained for total ERKs, with DAPI nuclear staining. C, qPCR for ELK1 target genes in Allstars-transfected and DVL3-depleted cells, mean ± SEM of triplicate independent analyses (*, P < 0.05, **, P < 0.01). D, Allstars or DVL3 (D) siRNA-transfected DU145 cells were treated with 120 nmol/L AZ12253801 for one hour and in the final 10 minutes with 10 nmol/L IGF1. Whole-cell extracts were analyzed by Western blot. DVL3 depletion enhanced IGF-induced ERK activation, and this effect was suppressed in AZ12253801-treated cells.

Figure 1. DVL3 mediates resistance to IGFIR inhibition. A, hits from second-round screens in DU145 cells, showing inhibition of cell viability (mean ± SEM log2 cell-surviving fraction), induced by single siRNAs (gray bars), siRNA pools (black), with Allstars (AS) control. Dashed line, 0.2 threshold for significant growth inhibition in AZ12253801-treated cells versus controls. B, isoform-specific qPCR showing mean ± SEM expression of DVLs 1–3 relative to GAPDH. Data from three independent experiments, each with three technical replicates. C, DU145 cells were transfected with 50 nmol/L Allstars siRNA or DVL3 siRNAs, 48 hours later treated with AZ12253801, and after 5 days assayed for viability. Graph, mean ± SEM viability expressed relative to solvent-treated controls; pooled data from three independent assays. Inset, Western blot to check DVL3 depletion. Graph to right, viability at 100 nmol/L AZ12253801 (***, P < 0.001, by one-way ANOVA). D, DU145 cells were transfected with isoform-specific DVL siRNAs, after 48 hours DVLs were measured by qPCR, normalized to GAPDH. Graph, mean ± SEM DVL3 expression relative to Allstars transfectants (n = 3). Table, AZ12253801 GI50 values in cells depleted of each DVL, fold sensitization calculated as ratio to GI50 in Allstars transfectants. E, DU145 cells infected with vectors encoding GFP or FLAG-DVL3 were transfected with 3’ UTR siRNA DVL3_6 and viability assayed after 5 days. Inset, Western blotting of parallel cultures. Right, graph, viability at 100 nmol/L AZ12253801 compared with Allstars control (**, P < 0.001, by t test). F, DU145 cells were transfected with Allstars, DVL3, and/or IGFIR or INSR siRNA (100 nmol/L total siRNA), and viability assayed after 5 days. Parallel cultures were collected after 60 hours to check target depletion. Compared with controls, depletion of IGFIR or DVL3 suppressed viability to 89% ± 2% and 73% ± 5%, respectively, and combined depletion to 38% ± 2% (***, P < 0.001, by one-way ANOVA), suggesting supraadditive growth inhibition. Viability in INSR-depleted cells was 88% ± 2% of control levels, without significant reduction (71% ± 4%) upon DVL3 depletion. G, DVL3 protein levels in 40 colorectal cancer cell lines previously assayed for figitumumab sensitivity, divided by growth inhibition induced by 10 μg/mL figitumumab into sensitive (>67% growth inhibition, n = 11) and resistant cells (moderately, 33%–67% inhibition, n = 8 or highly resistant, 0%–33% inhibition, n = 21). DVL3 protein levels were significantly lower in the figitumumab-sensitive cells (**, P = 0.003, by t test).

DVL3 Mediates Resistance to IGFIR Inhibition
whether intrinsic sensitivity to IGFIR inhibition is related to endogenous DVL3 expression. In prostate (n = 5) and breast cancer (n = 5) cell lines, there was no apparent relationship between endogenous DVL3 protein and AZ12253801 GI 50 (Supplementary Fig. S1D), perhaps reflecting genotypic differences in these small panels. In a larger sample (n = 40) of well-characterized colorectal cancer cell lines (23, 24), mean DVL3 protein levels were significantly lower in cell lines that were sensitive to IGFIR antibody figitumumab (n = 11), compared with cell lines that were moderately (n = 8) or highly (n = 21) figitumumab resistant (Fig. 1G), supporting the hypothesis that DVL3 protein is associated with resistance to IGFIR inhibition. There was no correlation with IGFIR expression (Supplementary Fig. S1E and S1F), consistent with the unchanged response to AZ12253801 in IGFIR-overexpressing PC3 prostate cancer cells (Supplementary Fig. S1A). We also noted lack of correlation between DVL3 protein and mRNA in the colorectal cancer cell lines, likely due to posttranscriptional regulation of DVL3 expression (25), and DVL3 mRNA did not associate with response to figitumumab (Supplementary Fig. S1G).

**Figure 3.** DVL3 depletion recapitulates effects of DVL3 depletion and sensitizes to AZ12253801 in vitro and in vivo. A, serum-starved DU145 cells were DVLi-treated for 16 hours, and whole-cell extracts were analyzed by Western blot using the indicated antibodies. DVLi inhibited DVL3 phosphorylation, reduced active β-catenin and phospho-S6, and increased levels of phospho-ERKs. B, DU145 whole-cell extract was treated with lambda phosphatase (P) in the absence or presence of phosphatase inhibitors (PI). The upper band of DVL3 immunoreactivity was abolished by λ phosphatase and restored by PI, consistent with DVL phosphorylation, as reported (22). C, DU145 cells were treated with AZ12253801 alone or with 100 μmol/L DVLi for 16 hours, and analyzed by Western blot for phospho-ERK. Bottom, paxillin and phospho-ERK were measured by Phos-tag gel. D, DU145 cells treated with 30 nmol/L AZ12253801 for 16 hours, and surviving colonies were fixed, stained, and counted. Graphs, mean ± SEM cell survival, expressed as survival relative to solvent-treated controls. E, DU145 cells treated with AZ12253801 alone (rate, 0.001, by t test), D, DU145 cells (left, 3,000 cells/well) and MCF-7 cells (right, 5,000 cells/well) were treated with AZ12253801 alone or with 50 μmol/L DVLi for 10 days, and surviving colonies were fixed, stained, and counted. Graphs, mean ± SEM cell survival, expressed as survival relative to solvent-treated controls. Legends show SF50 values derived from pooled data in three independent experiments (6 data points). E, DU145 cells were treated with DVLi alone or with 120 μmol/L AZ12253801. Graph, pooled data from three independent experiments. Right, graph, viability at 100 μmol/L DVLi (rate, 0.001, by the t test), F and G, prostate cancer (P) and breast cancer (G) cells were serum-starved, treated with 100 μmol/L DVLi for 16 hours, and analyzed by Western blot for phospho-ERK. Bottom, parallel cultures were treated with AZ12253801 alone or with 100 μmol/L DVLi. Table, pooled data from at least three independent CTG assays, showing G10 ratio (G10/IGFIR/G10/Comb), fold sensitization calculated as G10 ratio (G10/IGFIR/G10/Comb), H, male mice bearing DU145 xenografts were treated for 14 days with intraperitoneal solvent (0.05 mL DMSO), 25 mg/kg AZ12253801 twice daily, 50 mg/kg DVLi once daily, or the combination (DVLi + AZ12253801). Tumor growth in groups treated with AZ12253801 or DVLi was not significantly different from controls. The combination treatment group showed significant tumor growth retardation compared with groups treated with solvent (control, P < 0.001), AZ12253801 alone (P < 0.001), and DVLi alone (P < 0.01) by repeated measures ANOVA.

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The IGF-induced component of ERK activation in DVL3-depleted cells was effectively suppressed by AZ12253801 (Fig. 2D). These data suggest that DVL3 depletion enhances both basal and IGF-stimulated ERK activation, analogous to effects of PTEN loss on basal and ligand-induced AKT activation (28).

Of 12 candidate screen hits (Fig. 1A), five have known or putative roles as signaling regulators. In addition to DVL3, these include dual-specificity phosphatase 5 (DUSP5), connecter enhancer of kinase suppressor of RAS (CNKSRI), lemur tyrosine kinase 3 (LMTK3), and hormonally upregulated neu-associated kinase (HUNK), reported to regulate ERKs, RAS, AKT, and EGFR, respectively (29–32). Indeed, in DU145 cells, HUNK depletion enhanced IGF signaling to AKT (Supplementary Fig. S2A–S2C), supporting the concept that response to IGFIR inhibition is regulated by factors downstream of IGFIR.

Proximal WNT inhibition mimics effects of DVL3 depletion
Aiming to find a drug that recapitulates effects of DVL3 depletion, we tested compounds that block WNT signaling at different levels. Consistent with a role in Axin stabilization (33), the toolbox tankyrase inhibitor XAV939 upregulated Axin1 and inhibited mTOR, but did not activate ERKs or sensitize to AZ12253801 (Supplementary Fig. S3A–S3C). We then tested effects of WNT inhibition at a more proximal step, blocking DVL function directly. The DVL-PDZ domain has a peptide-binding cleft, involved in transducing signals from the membrane receptor Fz to downstream canonical and noncanonical pathways (22). The small-molecule inhibitor DVLi competitively binds to the cleft of the DVL-PDZ domain and blocks WNT signaling in Xenopus embryo and PC3 prostate cancer cells (34). This agent inhibited DVL3 phosphorylation and phenocopied DVL3 depletion, reducing active β-catenin and phospho-S6, activating ERKs in the absence of ligand, and sensitizing to AZ12253801 in viability and clonogenic assays (Fig. 3A–D). DVLi also sensitized to IGFIR TKI BMS-754807 that is being evaluated clinically (35; Supplementary Fig. S3D). We noted evidence for reciprocal sensitization: despite suppressing active β-catenin and mTOR, DVLi alone caused negligible growth inhibition, perhaps related at least in part to ERK activation, but with AZ12253801 caused significant loss of viability (Fig. 3E).

Although the DVLi we used has relatively low potency, the WNT pathway is an intense focus for drug development (34, 36), offering the prospect of more potent inhibitors in future. Therefore, we used this DVLi to perform proof-of-
principle experiments, testing its ability to influence sensitivity to IGFIR inhibition in the prostate and breast cancer cell lines we characterized previously (Supplementary Fig. S1D). Two prostate cancer cell lines (DU145 and 22Rv1) were sensitized to AZ12253801 by DVL inhibition; both had detectable basal ERK phosphorylation that was enhanced by DVLi (Fig. 3F). The other three (PC3, LNCaP, and LNCaP-LN3) did not manifest detectable basal or DVLi-induced ERK phosphorylation and were not sensitized to AZ12253801 by DVLi. The five breast cancer cell lines all showed increased ERK activation and enhanced response to AZ12253801 upon DVLi. The five breast cancer cell lines all showed increased ERK activation and enhanced response to AZ12253801 upon DVLi (Fig. 3G). Thus, 7 of 10 cell lines showed DVLi-induced ERK activation, and in each, DVLi enhanced response to IGFIR inhibition. Supporting the existence of functional cross-talk between the IGF axis and proximal WNT components, upregulation of IGF-binding protein 5 was shown to mediate growth inhibitory effects of a soluble Wnt inhibitor in murine MMTV-Wnt1-driven tumors. Therefore, to test the potential clinical relevance of our findings, we evaluated IGFIR and DVL inhibition in mice bearing DU145 prostate cancer xenografts. AZ12253801 or DVLi alone had no significant effects on tumor growth, but growth in the combination treatment group was significantly retarded compared with control-treated (P < 0.001), AZ12253801-treated (P < 0.001), and DVLi-treated animals (P < 0.01; Fig. 3H).

**DVL3 regulates IGF1-induced ERK activation**

These data indicate that blockade of proximal WNT signaling enhances sensitivity to IGFIR inhibition, and suggest that this property tracks with regulation of MEK–ERK signaling (Figs. 2A–C and 3F and G). To characterize this effect further, we performed time-course experiments in DVLi-inhibited DU145 cells. IGF1 induced rapid activation of IGFIR and AKT that persisted in control cells for at least 60 minutes, whereas ERK activation peaked at 10 minutes and resolved to basal levels by 60 minutes (Fig. 4A). In contrast, there was clear persistence of IGF1-induced ERK phosphorylation at 30 to 60 minutes in cells where DVL3 was inhibited (Fig. 4A) or depleted (Supplementary Fig. S4A). Persistent ERK activation in DVLi-inhibited cells was apparent within 4 hours of DVLi treatment, and was strikingly enhanced upon IGF1 treatment (Fig. 4B). An increase in IGF1-induced ERK activation was also observed in MCF7 cells (Supplementary Fig. S4B). These results support the existence of a link between the ability of DVL3 to influence sensitivity to IGFIR inhibition and to attenuate the response of the IGF axis to a mitogenic stimulus via MEK–ERK. Consequently, DVL3 depletion or inhibition creates an environment in whole-cell extracts. Bottom, graph, RAS activity in three independent assays, expressed relative to control (DVLi-untreated) cells, *P < 0.05, by t test. B, cartoon, IGFIR activates RAS via a protein complex that includes Shc, Grb2, and SOS. DU145 cells were transfected with Allstars (AS) siRNA or siRNAs to deplete IGFIR, Shc, Grb2, or SOS. After 48 hours, cells were treated with 100 μmol/L DVLi for 16 hours and analyzed by Western blotting. DVLi-induced ERK activation was abolished by depletion of Shc, SOS, or Grb2 but not IGFIR. C, DU145 whole-cell extracts were immunoprecipitated with control (IgG) or DVL3 antibodies and analyzed by Western blot, in parallel with DVL3 IP supernatant (s/n) to confirm DVL3 immunodepletion. DVL3 IPs contained DAB2 and Grb2 but not IGFIR. The same result was obtained in two further independent experiments. D, DU145 whole-cell extracts incubated with GST or GST–RAS–binding domain (RBD) of RAF, and pull-downs analyzed by Western blotting for activated (RBD-bound) RAS. Right, blot confirms RAS activation in each lane. E, DU145 cells were transfected with Allstars or DAB2 siRNA and after 48 hours, treated with AZ12253801 (left), and viability assayed after 5 days, showing GI50 values from three independent assays. Parallel cultures were analyzed by Western blot, shown to right.
that is permissive for signaling, recently characterized as "signalability" (37). In contrast, DVL inhibition did not influence the response to IGFIR in PC3 prostate cancer cells that were not sensitized to AZI1253801 by DVLi (Supplementary Fig. S1C). To test whether ERK activation is required for DVL3 to modify the response to IGFIR inhibition, we abolished ERK activity using MEK inhibitor AZD6244 (Fig. 4C). MEK-inhibited DU145 cells showed almost complete rescue from DVL3-induced sensitization to IGFIR inhibition (Fig. 4D), suggesting that the ability of DVL3 to regulate MEK–ERK contributes to the sensitization effect.

**DVL3 complexes with Shc, Grb2, and SOS to regulate RAS activation**

To investigate how DVL3 regulates ERKs, we performed RAS activation assays and found that RAS was activated in DVL-inhibited cells (Fig. 5A). This contrasts with previously reported WNT:ERK cross-talk, occurring at more distal WNT signaling nodes, that generates positive feedback between the two pathways (38). Given that DVL3 depletion did not activate IGFIR itself (Fig. 2A and Supplementary Fig. S4B), and DVL3-depleted or DVL-inhibited cells showed no change in EGF-induced ERK activation or sensitivity to EGFR inhibitor gefitinib (Supplementary Fig. S5A and S5B), we reasoned that RAS activation in DVL-depleted or DVL-inhibited cells was unlikely to be initiated at the level of these RTKs.

It is increasingly recognized that mitogenic signals generated by RTKs are integrated by complexes of adaptor and scaffolding proteins, including IRS-1, a well-recognized focus for feedback signaling via ERKs and mTOR-S6 kinase (39). However, IRS-1 knockdown did not influence AZI1253801 sensitivity or RAS activation induced by DVL inhibition (Supplementary Fig. S5C and S5D). In contrast, ERK activation in DVL-inhibited cells was suppressed by depletion of the adaptor protein Shc, exchange factor son-of-sevenless (SOS), or growth factor receptor--bound-2 (Grb2; Fig. 5B). ERK activation was not abolished by depletion of IGFIR (Fig. 5B), suggesting that the ability of DVL3 depletion or DVL inhibition to activate basal (ligand-unstimulated) MEK–ERK signaling was IGFIR independent, perhaps induced by another RTK or signaling complex.

These data suggest that DVL3 suppresses signal transduction at the level of the Shc–Grb2–SOS complex. DVL3 were not identified as Grb2 interactors in HEK293 cells (40), but DVL3 does contain the atypical proline-rich region shown in DVL2 to bind Grb2 and promote canonical WNT signaling (41). Indeed, complexes containing Grb2 and DVL3 were detectable in DU145 cells by immunoprecipitation and Grb2 pulldown (Fig. 5C and D). These complexes also contained the putative tumor suppressor Disabled 2 (DAB2), reported to limit RAS activation by competing with SOS for Grb2 binding (42–44). Consistent with this role, and with the association between ERK activation and sensitization to IGFIR inhibition (Fig. 3F and G and Fig. 4D), DAB2 depletion mimicked DVL3 depletion in sensitizing to AZI1253801 and enhancing IGF-induced ERK activation (Fig. 5E). DVL3 and DAB2 showed similar binding patterns to individual Grb2 domains (Supplementary Fig. S5F), interacting principally with the amino-terminal SH3 domain and central SH2 domain, whereas as reported, SOS bound to Grb2 amino and carboxy-terminal SH3 domains (45). We speculated that DVL3 interacts with Grb2 via DAB2, but found that DAB2-depleted cells still contained DVL3:Grb2 complexes (Supplementary Fig. S5F), and could be further sensitized to IGFIR inhibition by DVLi, with 18-fold reduction in AZI1253801 Glu in DAB2-deleted, DVL3-inhibited cells, compared with 2.8- and 4.6-fold sensitization induced separately by DAB2 depletion or DVL inhibition (Fig. 5F). These data suggest that DVL3 has a different role from DAB2 in regulating signal transduction to RAS.

**DVL3 expression in clinical cancers**

To investigate the clinical relevance of these findings, we evaluated DVL3 expression in patient tumors. Given the lack of correlation shown previously between DVL3 mRNA and protein (Supplementary Fig. S1G), we developed an IHC protocol, with controls including DVL3-depleted DU145 cells, to assess DVL3 expression by intensity × percentage score (IPS; Supplementary Methods; Fig. 6A and Supplementary Fig. S6A and S6B). Approximately 50% of breast and prostate cancers contained moderate or heavy cytoplasmic DVL3 (Supplementary Fig. S6C) that did not correlate with stage, grade, or patient survival (Supplementary Table S7). As a first approach to testing for the correlation between DVL3 protein and sensitivity to IGFIR inhibition, we assessed DVL3 in Ewing sarcomas, reported to be responsive to IGFIR inhibitor monotherapy (4, 5, 46, 47), and head and neck squamous cell cancers (HNSCC) that are resistant (48). The results are shown in Fig. 6B and C; all but one of the Ewing sarcomas contained low or no detectable DVL3, and the mean DVL3 staining score was significantly higher in HNSCCs (IPS = 0.0032). We then assessed DVL3 protein expression in archival tumors from patients recruited to early-phase clinical trials of IGFIR antibodies figitumumab or AVE164 (Supplementary Table S8; refs. 9, 48). Figure 6D shows examples of DVL3 staining in eight of the trial cases, and Fig. 6E shows analysis with respect to progression-free survival (PFS). Although there was overlap in DVL3 expression between patients experiencing early progression versus prolonged control, it was notable that of eight patients achieving prolonged disease control (>84 days, including one partial remission), six of these tumors had low DVL3 protein expression (IPS <5; Supplementary Table S8). PFS was longer in patients whose tumors showed no/low DVL3 (IPS, 137 ± 28 days) compared with patients whose tumors had moderate or strong DVL3 (IPS, 70 ± 13 days; P = 0.031), and DVL3 staining was negatively correlated with PFS (Spearman r = −0.55; P = 0.0125; Fig. 6E). Included here were melanomas, ovarian, gastrointestinal, and HNSCCs (Supplementary Table S8), suggesting that DVL3 expression may have predictive value for response to IGFIR inhibition in a range of tumor types.

These data define new roles for DVL3 in suppressing signal transduction from IGFIR to RAS, and regulating response to IGFIR blockade. The association between RAS...
activation and sensitization to IGFIR inhibition is counterintuitive, but is consistent with a report that KRAS-mutated non-small cell lung cancer cells show IGF-dependent PI3K activity and are sensitive to IGFIR inhibition (49). The finding that responses to IGFIR inhibition are modified by manipulating DVL3 expression or function suggests that DVL3 contributes to the resistance phenotype. Thus, DVL3 depletion or inhibition leads to amplification of IGF-induced RAS–MEK–ERK activation, and this property is associated with enhanced sensitivity to IGFIR inhibition (Fig. 7). Similar AZ12253801 sensitization and ERK activation were induced by depletion of DAB2 (Fig. 5E), reported to interact with Grb2 and suppress RAS activation (42–44). DAB2-depleted cells were capable of responding to DVL inhibition with enhanced ERK activation and AZ12253801 sensitization (Fig. 5F), suggesting that DVL3 has a nonredundant function in the Shc–Grb2–SOS complex.

Our screen identified additional candidate resistance mediators (DVL3, DUSP5, CNKS1, LMTK3, and HUNK) with roles in regulating receptor or postreceptor signaling. The ability to influence IGFIR sensitivity to both ligand and receptor inhibition is reminiscent of the functional effects of EGFR kinase mutations, which favor the active conformation of EGFR kinase and render EGFR sensitive to EGFR TKIs (50). Comparable mutations have been sought but not reported in IGFIR; it is plausible that similar tuning of IGF signaling is achieved by changes in signal transduction complexes downstream of IGFIR. In summary, our data identify a role for DVL3 in suppressing signal transduction from IGFIR to RAS and attenuating response to IGFIR blockade. We propose that the

Figure 7. Model for DVL3 as a regulator of RAS activation and mediator of resistance to IGFIR inhibition. DVL3 exists in a complex of adaptor proteins that includes Shc, Grb2, and the tumor suppressor DAB2. Both DVL3 and DAB2 suppress signal transduction from IGFIR to RAS. When DVL3 and/or DAB2 are depleted, or DVLs are inhibited, there is enhanced basal (ligand-unstimulated) RAS–MEK–ERK activation, and in the presence of ligand, selective amplification of IGF signaling via the RAS–MEK–ERK pathway. This state is associated with increased dependency on IGF signaling and enhanced sensitivity to IGFIR inhibition.
proportion of potentially responsive patients in IGFIR trials could be significantly increased by selecting cases with low-DVL3 tumors.

Disclosure of Potential Conflicts of Interest

T.M. Jones received a commercial research grant from GSK. V.M. Macaulay is a consultant/advisory board member for Consultancy. No potential conflicts of interest were disclosed by the other authors.

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DVL3 Mediates Resistance to IGFIR Inhibition


Dsh Homolog DVL3 Mediates Resistance to IGFIR Inhibition by Regulating IGF-RAS Signaling

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