Dsh homolog DVL3 mediates resistance to IGF-1R inhibition by regulating IGF-RAS signalling

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Conflict of interest statement: VMM and SG have received research grants from AstraZeneca. The other authors have no conflict of interest.

Running title: DVL3 mediates resistance to IGF-1R inhibition

Key words: IGF-1R, IGF-1R inhibitor, DVL3, RAS, biomarker

Abstract

Drugs that inhibit IGF-1 receptor IGF-1R 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 IGF-1R inhibition. Sensitivity to IGF-1R inhibition was enhanced specifically in vitro and in vivo by genetic or pharmacological 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 IGF-1R to RAS, that includes Shc, Grb2, SOS and the tumor suppressor DAB2. Dual DVL and DAB2 blockade synergized in activating ERKs and sensitizing cells to IGF-1R inhibition, suggesting a non-redundant role for DVL3 in the Shc-Grb2-SOS complex. Clinically, tumors that responded to IGF-1R 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 IGF-1R antibodies. Since IGF-1R 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 post-receptor level through adaptor protein complexes, influencing cellular dependence on the IGF axis and identifying a patient population with potential to benefit from IGF-1R inhibition.
Introduction

Type 1 insulin-like growth factor receptor (IGF-1R) 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 pro-tumorigenic signal (2, 3). In early clinical trials, IGF-1R 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 3 benefit in unselected patients (10). Clearly, a better understanding of IGF-1R biology is required if this therapeutic approach is to be successful. Key priorities are to understand what makes tumors resistant to IGF-1R 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 IGF-1R antagonist.
Materials and Methods

Cell lines, 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, Hertfordshire UK), 22Rv1 prostate cancer cells from the American Type Culture Collection, and BT20 and BT549 from Dr Anthony Kong, University of Oxford. All cell lines were mycoplasma-free when tested with MycoAlert (Lonza Rockland Inc, Rockland US). 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'-CTCGAGTCACATCACATCCACAAAGAACT-3' incorporating BamH1 and Xho1 sites respectively (underlined), digested with BamH1 and Xho1 (New England Biolabs), cloned into BamHI-XhoI 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 (12), Supplementary Methods, and Table S1.

siRNA screens

Screens were performed as (13, 14), using kinase siRNA library (siARRAY, targeting 779 known and putative human protein kinases; Dhharmacon) 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 50nM 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 AZ1223580 at the GI50 for 5 days, and viability was assessed by CellTiter Glo (CTG) assay (Promega). Duplicate primary screens were analyzed to derive Z’ factors (dynamic range) and Z-scores (effect of siRNA on viability, corrected for within/between plate variation), as described (14-16). Triplicate second round screens used four individual siRNAs and the 4
siRNAs pooled; these and additional siRNAs are listed in Table S2. Data were analyzed to generate log2 surviving fractions as the final score for the effect of IGF-1R inhibition on viability; scores < -0.2 were regarded as significant, as described (13, 14). Hits were validated by siRNA-transfection as above, analyzing after 48 hours by western blot or quantitative reverse-transcription PCR (qRT-PCR, with primers listed in Table S3), or treating with solvent or AZ12253801 and assaying for viability after 5 days or clonogenic survival after 10-20 days. Pooled data from ≥3 independent experiments were curve-fitted using GraphPad Prism v5 to interpolate GI50 and SF50 (concentrations inhibiting 50% of growth or survival).

Immunofluorescence, immunohistochemistry

Cells underwent immunofluorescent staining using ERK antibody ( nº4695, Cell Signaling Technology). IHC used DVL3 antibody 4D3 (Santa Cruz; see Supplementary Methods).

Xenografts

As detailed in Supplementary Methods, mice bearing DU145 xenografts were randomly allocated to 14 days treatment with 0.05ml solvent (DMSO), 25mg/kg AZ12253801 twice daily, 50mg/kg DVLi once daily or combination treatment.
Results and Discussion

**siRNA screens for sensitization to IGF-1R inhibition**

IGF-1R over-expression occurs frequently in common cancers, but is a poor predictor of clinical sensitivity to IGF-1R inhibition (10). Consistent with this, IGF-1R over-expression in PC3 prostate cancer cells did not influence response to IGF-1R tyrosine kinase inhibitor (TKI) AZ12253801 that has ~10-fold selectivity over the insulin receptor (INSR; (17); Figure S1A). To identify proteins that influence response to AZ12253801, we performed siRNA screens in DU145 prostate cancer and MCF7 breast cancer cells, confirming that AZ12253801 inhibited IGF-1R phosphorylation and cell viability (Figure S1B). Primary screens were performed to deplete ~1000 targets; given our interest in the involvement of IGF-1R kinase in the DNA damage response (12, 18), we selected siRNA libraries targeting kinase-related and DNA repair-associated proteins, with positive (siPLK1) and negative (Allstars) control siRNAs, as described (13, 14). Forty-eight hours after siRNA transfection to allow target depletion, cells were treated with solvent or AZ12253801 at the GI50 and viability was assayed 5 days later. Duplicate DU145 screens were highly reproducible (R² values ≥0.8) and sensitive, with Z-factors of 0.23-0.6 (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 (Table S5). Triplicate second-round screens identified 12 putative resistance mediators, including regulators of the cell cycle and DNA damage response, and proteins with poorly-characterized functions (Figure 1A). Seven hits in DU145 screens (CDKN2C, CNKSR1, DUSP5, HUNK, LMTK3, MPP2, DVL3) were also candidate hits in MCF7 cells. Validation in low-throughput format confirmed that depletion of each hit enhanced AZ12253801 sensitivity (Table S6).

**DVL3 mediates resistance to IGF-1R 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 3 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 (Figure 1B), and depletion of DVL3 but not DVL 1 or 2 sensitized to AZ12253801 (Figure 1C,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 (Figure 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. While DVL3-depleted DU145 and MCF7 cells were sensitized to IGF-1R depletion, INSR-depleted cells showed no significant reduction in viability upon DVL3 depletion (Figures 1F, S1C), supporting the contention that functional interaction between DVL3 and AZ12253801 is related to the ability of AZ12253801 to block IGF-1R. We next tested whether intrinsic sensitivity to IGF-1R 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 GI50 (Figure S1D), perhaps reflecting genotypic differences in these small panels. In a larger sample (n=40) of well-characterized colorectal cancer (CRC) cell lines (23, 24), mean DVL3 protein levels were significantly lower in cell lines that were sensitive to IGF-1R antibody figitumumab (n=11), compared with cell lines that were moderately (n=8) or highly (n=21) figitumumab resistant (Figure 1G), supporting the hypothesis that DVL3 protein is associated with resistance to IGF-1R inhibition. There was no correlation with IGF-1R expression (Figure S1E-F), consistent with the unchanged response to AZ12253801 in IGF-1R overexpressing PC3 prostate cancer cells (Figure S1A). We also noted lack of correlation between DVL3 protein and mRNA in the CRC cell lines, likely due to post-transcriptional regulation of DVL3 expression (25), and DVL3 mRNA did not associate with response to figitumumab (Figure S1G).

**DVL3 depleted cells show enhanced ERK activation**

We speculated that the association between DVL3 and resistance to IGF-1R inhibition may reflect cross-talk between the WNT and IGF pathways. Previously, we and others showed that the adaptor protein IRS-1 undergoes IGF-induced interaction with β-catenin, promoting β-catenin stabilization and
transcriptional activity (19, 20). However, in DU145 cells there was no evidence that IGF-1 affected levels of active (non-phosphorylated) β-catenin or phosphorylated DVL3 (Figure 2A). DVL3 depletion suppressed active β-catenin and phosphorylation of mTOR effector S6, consistent with known roles for DVL3 in canonical WNT signalling and mTOR activation (22, 26). DVL3-depleted cells showed no change in IGF-1R expression or activation, but unexpectedly showed increased MEK-ERK phosphorylation (Figure 2A). Quantification of these data indicates that DVL3 depletion had a detectable (although not statistically significant) effect on basal (ligand-unstimulated) ERK activation, and significantly enhanced IGF-induced ERK activation. This effect was accompanied by ERK nuclear translocation and up-regulation of ERK-ELK target genes (27); Figure 2B,C). The IGF-induced component of ERK activation in DVL3-depleted cells was effectively suppressed by AZ12253801 (Figure 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 hits identified in our screen (Figure 1A), five have known or putative roles as signalling regulators: dual specificity phosphatase 5 (DUSP5), connector enhancer of kinase suppressor of RAS (CNKSR1), 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 signalling to AKT (Figure S2A-C), supporting the concept that response to IGF-1R inhibition is regulated by factors downstream of IGF-1R.

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 signalling 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 (Figure S3A-C). 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 non-canonical pathways (22). The small molecule inhibitor DVL-PDZ inhibitor II (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 (Figure 3A-D). DVLi also sensitized to IGF-1R TKI BMS-754807 that is being evaluated clinically (35); Figure 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 (Figure 3E).

While 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 IGF-1R inhibition in the prostate and breast cancer cell lines we characterized previously (Figure S1D). Two prostate cancer cell lines (DU145 and 22Rv1) were sensitized to AZ12253801 by DVLi inhibition; both had detectable basal ERK phosphorylation that was enhanced by DVLi (Figure 3F). The other three (PC3, LNCaP, LNCaP-LN3) did not manifest detectable basal or DVLi-induced ERK phosphorylation and were not sensitized to AZ12253801 by DVLi. The 5 breast cancer cell lines all showed increased ERK activation and enhanced response to AZ12253801 upon DVLi inhibition (Figure 3G). Thus 7 of 10 cell lines showed DVLi-induced ERK activation and in each, DVLi enhanced response to IGF-1R inhibition.

Supporting the existence of functional cross-talk between the IGF axis and proximal WNT components, up-regulation of IGF binding protein 5 was shown to mediate growth inhibitory effects of a soluble Wnt inhibitor in murine MMTV-Wnt1-driven tumors (36). Therefore, to test the potential clinical relevance of our findings, we evaluated IGF-1R and DVLi 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; Figure 3H).

**DVL3 regulates IGF-1-induced ERK activation**

These data indicate that blockade of proximal WNT signalling enhances sensitivity to IGF-1R inhibition, and suggest that this property tracks with regulation of MEK-ERK signalling (Figures 2A-C, 3F,G). To characterize this effect further, we performed time-course experiments in DVL-inhibited DU145 cells. IGF-1 induced rapid activation of IGF-1R and AKT that persisted in control cells for at least 60min, while ERK activation peaked at 10min and resolved to basal levels by 60min (Figure 4A). In contrast, there was clear persistence of IGF-induced ERK phosphorylation at 30-60min in cells where DVL3 was inhibited (Figure 4A) or depleted (Figure S4A). Persistent ERK activation in DVL-inhibited cells was apparent within 4hr of DVLi treatment, and was strikingly enhanced upon IGF-treatment (Figure 4B). An increase in IGF-induced ERK activation was also observed in MCF7 cells (Figure S4B). These results support the existence of a link between the ability of DVL3 to influence sensitivity to IGF-1R 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 that is permissive for signalling, recently characterized as ‘signalability’ (37). In contrast, DVL inhibition did not influence the response to IGF-1 in PC3 prostate cancer cells that were not sensitized to AZ12253801 by DVLi (Figure S4C). To test whether ERK activation is required in order for DVL3 to modify the response to IGF-1R inhibition, we abolished ERK activity using MEK inhibitor AZD6244 (Figure 4C). MEK-inhibited DU145 cells showed almost complete rescue from DVLi-induced sensitization to IGF-1R inhibition (Figure 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 (Figure 5A). This contrasts with previously reported WNT:ERK cross-talk
occurring at more distal WNT signalling nodes, that generates positive feedback between the two pathways (38). Given that DVL3 depletion did not activate IGF-1R itself (Figures 2A, S4B), and DVL3-depleted or DVL-inhibited cells showed no change in EGF-induced ERK activation or sensitivity to EGFR inhibitor gefitinib (Figure S5A,B), we reasoned that RAS activation in DVL depleted or 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 signalling via ERKs and mTOR-S6 kinase (39). However, IRS-1 knockdown did not influence AZ12253801 sensitivity or ERK activation induced by DVL inhibition (Supplementary Figure S5C,D). 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; Figure 5B). ERK activation was not abolished by depletion of IGF-1R (Figure 5B), suggesting that the ability of DVL3 depletion or DVL inhibition to activate basal (ligand-unstimulated) MEK-ERK signalling was IGF-1R independent, perhaps induced by another RTK or signalling complex.

These data suggest that DVL3 suppresses signal transduction at the level of the Shc-Grb2-SOS complex. DVLs 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 (Figure 5C, 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 IGF-1R inhibition (Figure 3F,G, Figure 4D), DAB2 depletion mimicked DVL3 depletion in sensitizing to AZ12253801 and enhancing IGF-induced ERK activation (Figure 5E). DVL3 and DAB2 showed similar binding patterns to individual Grb2 domains (Figure S5E), interacting principally with the amino-terminal SH3 domain and
central SH2 domain, while 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 (Figure S5F), and could be further sensitized to IGF-1R inhibition by DVLi, with 18-fold reduction in AZ12253801 GI\textsubscript{50} in DAB2-deleted, DVL inhibited cells, compared with 2.8 and 4.6 fold sensitization induced separately by DAB2-depletion or DVL-inhibition (Figure 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 (Figure S1G), we developed an immunohistochemistry protocol, with controls including DVL3-depleted DU145 cells, to assess DVL3 expression by intensity x percentage score (IPS; Supplementary Methods; Figure 6A S6A, B). Approximately 50% of breast and prostate cancers contained moderate or heavy cytoplasmic DVL3 (Figure S6C) that did not correlate with stage, grade or patient survival (Table S7). As a first approach to testing for correlation between DVL3 protein and sensitivity to IGF-1R inhibition, we assessed DVL3 in Ewing sarcomas, reported to be responsive to IGF-1R inhibitor monotherapy (4, 5, 46, 47), and head and neck squamous cell cancers (HNSCC) that are resistant (48). The results are shown in Figure 6B, C; all but one of the Ewing sarcomas contained low or no detectable DVL3, and the mean DVL3 staining score was significantly higher in HNSCC (p=0.0033). We then assessed DVL3 protein expression in archival tumors from patients recruited to early phase clinical trials of IGF-1R antibodies figitumumab or AVE164 (9, 48; Table S8). Figure 6D shows examples of DVL3 staining in 8 of the trial cases, and Figure 6E shows analysis with respect to progression-free survival (PFS). While there was overlap in DVL3 expression between patients experiencing early progression vs prolonged control, it was notable that of 8 patients achieving prolonged disease control (>84 days, including one partial remission), 6 of these tumors had low DVL3 expression (IPS <5; Table S8). PFS was longer in patients whose tumours showed no/low DVL3 (n=9, PFS 137 ± 28 days) compared with patients whose tumors had moderate or
strong DVL3 (n=11, PFS 70 ± 13 days, p = 0.031) and DVL3 staining was negatively correlated with PFS (Spearman r = -0.55, p = 0.0125; Figure 6E). Included here were melanomas, ovarian, gastrointestinal and HNSCC (Table S8), suggesting that DVL3 expression may have predictive value for response to IGF-1R inhibition in a range of tumor types.

These data define new roles for DVL3 in suppressing signal transduction from IGF-1R to RAS, and regulating response to IGF-1R blockade. The association between RAS activation and sensitization to IGF-1R inhibition is counter-intuitive, but is consistent with a report that KRAS-mutated NSCLC cells show IGF-dependent PI3K activity and are sensitive to IGF-1R inhibition (49). The finding that responses to IGF-1R 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 IGF-1R inhibition (Figure 7). Similar AZ12253801 sensitization and ERK activation was induced by depletion of DAB2 (Figure 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 (Figure 5F), suggesting that DVL3 has a non-redundant function in the Shc-Grb2-SOS complex.

Our screen identified additional candidate resistance mediators (DVL3, DUSP5, CNKSR1, LMTK3 and HUNK) with roles in regulating receptor or post-receptor signaling. The ability to influence IGF-1R 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 IGF-1R; it is plausible that similar tuning of IGF signalling is achieved by changes in signal transduction complexes downstream of IGF-1R.

In summary, our data identify a role for DVL3 in suppressing signal transduction from IGF-1R to RAS and attenuating response to IGF-1R blockade. We propose that the proportion of potentially-
responsive patients in IGF-1R trials could be significantly increased by selecting cases with low-DVL3 tumors.

Disclosure of Potential Conflicts of Interest

This research was supported by a grant from AstraZeneca; aside from this we have no conflict of interest.

Acknowledgements

This work was supported by the NIHR Oxford Biomedical Research Centre, Oxford Experimental Cancer Medicine Centre (ECMC), HEFCE Clinical Senior Lectureship to VMM, and research grants from UCARE-Oxford, Breast Cancer Campaign, Molecular and Cellular Medicine Board of MRC and AstraZeneca. Research in the laboratory of WFB is funded by Roche/Glycart. SQA is funded by an Academy of Medical Sciences Clinical Lecturer Starter grant and support from the Nuffield Department of Surgical Sciences (Oxford University Hospitals National Health Service Trust, Oxford). JYB is supported by grants Netsarc-LYRIC_4664 and EuroSarc (FP7 278472). We are grateful to Elaine Kilgour, Elizabeth Anderson and Jon Curwen at AstraZeneca for providing AZ12253801 and advice on its use, James Christensen at Pfizer for providing figitumumab, Ludwig Van den Hove at Pfizer and John Dahlquist at ImmunoGen for permission to use clinical data from the GORTEC-2008-2 and TED6421 trials respectively, Divita Jata, Oxford Centre for Histopathology Research, and Olga Perestenko, Department of Oncology, Weatherall Institute of Molecular Medicine for assistance with DVL3 IHC, Antoinette Wetterwald, University of Bern, for assistance with the prostate TMA, and Anderson Ryan and Bass Hassan for comments on the manuscript. We gratefully acknowledge the contribution of our co-author Takeshi Kashima, who died while this manuscript was under review.
References


Legends to Figures

Figure 1. DVL3 mediates resistance to IGF-1R 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 (grey bars), siRNA pools (black), with Allstars (AS) control.  Dashed line represents -0.2 threshold for significant growth inhibition in AZ12253801-treated cells vs controls.  

B) Isoform-specific qPCR showing mean ± SEM expression of DVLs 1-3 relative to GAPDH.  Data from three independent experiments, each with 3 technical replicates.  

C) DU145 cells were transfected with 50nM Allstars (AS) siRNA or DVL3 siRNAs, 48hr later treated with AZ1253801, and after 5 days assayed for viability.  

Graph: mean ±SEM viability expressed relative to solvent-treated controls, pooled data from 3 independent assays.  Inset: western blot to check DVL3 depletion.  

Graph to right: viability at 100nM AZ12253801 (***p<0.001 by one-way ANOVA).  

D) DU145 cells were transfected with isoform-specific DVL siRNAs, after 48hr DVLs were measured by qPCR, normalized to GAPDH.  Graph shows mean ± SEM DVL3 expression relative to Allstars (AS) 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.  

Graph to right: viability at 100nM AZ12253801 (*p<0.05, **p<0.01 by one-way ANOVA).  

F) DU145 cells were transfected with Allstars (AS), DVL3 and/or IGF-1R or INSR siRNA (100nM total siRNA) and viability assayed after 5 days.  Parallel cultures collected after 60hr to check target depletion.  Compared with controls, depletion of IGF-1R 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 supra-additive 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 CRC cell lines previously assayed for figitumumab sensitivity, divided by growth inhibition induced by 10µg/ml figitumumab into sensitive (>67% growth inhibition, n=11) and resistant (moderately, 33-67% inhibition, n=8 or highly resistant, 0-
DVL3 protein levels were significantly lower in the figitumumab sensitive cells (**p=0.003 by t-test).

**Figure 2. DVL3 depletion enhances IGF signaling via MEK-ERK.** A) DU145 cells were Allstars (AS) or DVL3 siRNA-transfected and after 48hr treated with 10nM IGF-1 for 10min. Graphs to right: mean ± SEM phospho-IGF-1R, phospho-AKT, phospho-MEK and phospho-ERKs corrected for equivalent total proteins, expressed as % IGF-treated Allstars-transfectants from 3 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 12hr 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 (AS) or DVL3 (D) siRNA-transfected DU145 cells were treated with 120 nM AZ12253801 for one hour and in the final 10min with 10nM IGF-1. 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 3. DVL inhibition 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 lamda phosphatase (λ) 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 100µM DVLi. Legend shows GI₅₀ values derived from pooled data in 3 independent experiments. Graph to right: viability at 30nM AZ12253801 (**p<0.001 by t-test). D) DU145 cells (left, 3000 cells/well) and MCF-7 cells (right, 5000 cells/well) were treated with AZ12253801 alone or with AZ12253801 and 50µM DVLi for 10 days, and surviving colonies were fixed, stained and counted.
Graphs show mean ± SEM cell survival, expressed as survival relative to solvent-treated controls. Legends show SF_{50} from pooled data in 2 independent experiments (6 data points). E) DU145 cells were treated with DVLi alone or with 120nM AZ12253801. Graph shows pooled data from 3 independent experiments. Graph to right: viability at 100nM AZ12253801 (**p<0.001 by t-test).

F) Prostate cancer and G) and breast cancer cells were serum-starved, treated with 100µM DVLi for 16hr and analyzed by western blot for phospho-ERK. Lower: parallel cultures were treated with AZ12253801 alone or with 100µM DVLi. Table: pooled data from at least 3 independent CTG assays, showing GI_{50} (nM) for AZ1225380 alone (IGF-1Ri) or with DVLi (Comb), fold sensitization calculated as GI_{50} ratio (GI_{50} IGF-1Ri / GI_{50} Comb).

H) Male mice bearing DU145 xenografts were treated for 14 days with intraperitoneal solvent (0.05ml DMSO), 25mg /kg AZ12253801 twice daily, 50mg/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.

**Figure 4. The ability of DVL3 to influence sensitivity to IGF-1R inhibition is linked to its capacity to regulate ERK activation.** A) Serum-starved DU145 cells were treated with 100µM DVLi for 16hr and in the final 0-60min with 10nM IGF-1. B) Serum-starved DU145 cells were treated with 100µM DVLi for 1-16hr and in the final 30min with 10nM IGF-1. Graphs below A) and B) show phospho-ERK (mean ± range), corrected for total ERK, from two independent experiments. C) Serum-starved DU145 cells were treated with 100µM DVLi and/or 5µM AZD6244 for 16hr and in the final 30min with 10nM IGF-1. D) DU145 cells were treated with AZ12253801 alone or with 100µM DVLi alone or with 5µM MEK inhibitor AZD6244, and cell viability was assayed after 5 days. Graph shows pooled data from 4 independent experiments, legend shows GI_{50} values and fold sensitization (GI_{50} ratio). Graph to right: relative proliferation at 100nM AZ12253801 (**p<0.01, ***p<0.001 by one-way ANOVA).
Figure 5. DVL3 regulates signaling from IGF-1R to RAS. A) DU145 cells were treated with 100μM DVLi for 16hr, lysates were incubated with GST or GST-RAS binding domain (RBD) of RAF, and pull-downs analyzed by western blotting for activated (RBD-bound) RAS. Blot to right confirms ERK activation in whole cell extracts. Graph below: RAS activity in three independent assays, expressed relative to control (DVLi-untreated) cells, *p<0.05 by t-test. B) Cartoon: IGF-1R activates RAS via a protein complex that includes Shc, Grb2 and SOS. DU145 cells were transfected with Allstars (AS) siRNA or siRNAs to deplete IGF-1R, Shc, Grb2 or SOS. After 48hr, cells were treated with 100μM DVLi for 16hr and analyzed by western blotting. DVLi-induced ERK activation was abolished by depletion of Shc, SOS or Grb2 but not IGF-1R. 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 IGF-1R. The same result was obtained in two further independent experiments. D) DU145 whole cell extracts incubated with GST or GST-Grb2, and precipitated proteins analyzed by western blot, revealing the presence of DVL2, DVL3, Shc and DAB2 in Grb2 pull-downs. Representative of three independent experiments. E) DU145 cells were transfected with Allstars (AS) or DAB2 siRNA and after 48hr: left, treated with AZ12253801 and viability assayed after 5 days, showing GI50 values from 3 independent assays; right, serum-starved overnight and treated with 10nM IGF-1 for 10min. DAB2 knockdown sensitized to AZ12253801 and also activated ERKs. F) Allstars-transfected or DAB2-depleted cells were treated with 100μM DVLi and/or AZ12253801 and cell viability assayed after 5 days. Graph: mean ± SEM viability, pooled data from 3 independent assays. Legend shows GI50 values and fold sensitization to AZ12253801. Parallel cultures were analyzed by western blot, shown to right.

Figure 6. DVL3 protein expression is inversely correlated with response to IGF-1R antibody. A) DU145 cells were transfected with Allstars or DVL3 siRNA, after 48hr were formalin-fixed, paraffin-embedded and used as controls for immunostaining, in parallel with sections of a TURP in which 100% of...
chippings were involved by Gleason grade 4+5 prostate cancer from a previously-untreated patient. These cell and tissue controls were included in every staining run for quality control purposes. B) Tissue microarrays of Ewing sarcoma and HNSCC underwent IHC staining in the same staining run. Representative images of: upper: Ewing sarcoma; to the left is a sample of normal kidney included as a control on the same TMA slide; lower: HNSCC. Most of the Ewing sarcomas had light or no DVL3 signal; intense focal signal in one tumor may be due to staining of macrophages. C) DVL3 IPS scores for 9 cases of Ewing sarcoma and 24 cases of HNSCC, showing significantly higher mean score in HNSCC. D) Examples of DVL3 IHC on tumors from patients on IGF-1R antibody trials, showing diagnosis, progression-free survival (PFS, days) and DVL3 Intensity x Percentage Score (IPS). E) Graph of progression-free survival (PFS) vs DVL3 IPS in 20 patients treated on IGF-1R antibody trials.

Figure 7. Model for DVL3 as a regulator of RAS activation and mediator of resistance to IGF-1R 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 IGF-1R 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 IGF-1R inhibition.
Figure 2

A

B

C

D
Figure 3

A

B

C

D

E

F

G

H

Author Manuscript Published OnlineFirst on August 28, 2014; DOI: 10.1158/0008-5472.CAN-14-0806
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Figure 4

A

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<td>β-tubulin</td>
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Relative phospho-ERK1/2

B

| IGF-1 (min) | 0 | 1 | 16 | 30 |
| DVLi (hr)   | 0 | 1 | 4  | 16 |
| DVL3        |    |   |    |    |
| P-IGF-1R    |    |   |    |    |
| IGF-1R      |    |   |    |    |
| P-AKT       |    |   |    |    |
| P-ERK1/2    |    |   |    |    |
| ERK 1/2     |    |   |    |    |
| β-tubulin   |    |   |    |    |

Relative phospho-ERK1/2

C

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D

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Relative cell proliferation

**NS**

**NS**

**NS**
Figure 7

**左侧：**
- Wnts
- Frizzled
- LRP5/6
- Dsh/DVL
- β-catenin degradation
- Axin-APC-GSK3β
- β-catenin
- PI3K
- AKT
- mTOR
- IRS-1
- RAS-GDP
- RAS-GTP
- Cyclin D1
- TCF-LEF

**中央：**
- DVL3, DAB2 present: - suppress ability of IGFs to activate RAS
- Reduced dependence on IGF signalling
- Resistant to IGF-1R inhibition

**右侧：**
- IGF-1
- IGF-1R
- Shc
- Grb2
- SOS
- RAS-GDP
- RAS-GTP
- Lack of DVL3, DAB2:
  - ↑ basal MEK-ERK activity
  - ↑ signal transduction from IGF-1R to RAS
- Cyclin D1, FOS
- TAP, TBP
- ELK
- Increased dependence on IGF signalling
- Sensitive to IGF-1R inhibition
Dsh homolog DVL3 mediates resistance to IGF-1R inhibition by regulating IGF-RAS signalling

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Cancer Res  Published OnlineFirst August 28, 2014.