

A functional RNAi screen for regulators of receptor tyrosine kinase and ERK signalling

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Receptor tyrosine kinase (RTK) signalling through extracellular-signal-regulated kinases (ERKs) has pivotal roles during metazoan development, underlying processes as diverse as fate determination, differentiation, proliferation, survival, migration and growth. Abnormal RTK/ERK signalling has been extensively documented to contribute to developmental disorders and disease, most notably in oncogenic transformation by mutant RTKs¹ or downstream pathway components such as Ras and Raf². Although the core RTK/ERK signalling cassette has been characterized by decades of research using mammalian cell culture and forward genetic screens in model organisms, signal propagation through this pathway is probably regulated by a larger network of moderate, context-specific proteins. The genes encoding these proteins may not have been discovered through traditional screens owing, in particular, to the requirement for visible phenotypes. To obtain a global view of RTK/ERK signalling, we performed an unbiased, RNA interference (RNAi), genome-wide, high-throughput screen in *Drosophila* cells using a novel, quantitative, cellular assay monitoring ERK activation. Here we show that ERK pathway output integrates a wide array of conserved cellular processes. Further analysis of selected components—in multiple cell types with different RTK ligands and oncogenic stimuli—validates and classifies 331 pathway regulators. The relevance of these genes is highlighted by our isolation of a Ste20-like kinase and a PPM-family phosphatase that seem to regulate RTK/ERK signalling *in vivo* and in mammalian cells. Novel regulators that modulate specific pathway outputs may be selective targets for drug discovery.

Genome-wide RNAi screens can identify novel components of signal transduction pathways (for example, see ref. 3). The outputs from traditional transcriptional pathway reporters may be biased by cell type, integrate unknown additional pathways, and not linearly reflect endogenous signalling. In contrast, we developed a phospho-specific antibody reporter of proximal, endogenous pathway activity. Monoclonal antibodies recognizing the dually phosphorylated, active form of mammalian ERK1/2 (dpERK) have been characterized to report the dynamic activation of the single *Drosophila* ERK isoform, Rolled, downstream of multiple RTKs^{4,5}.

Drosophila haemocytes express the insulin receptor (InR), which activates both the Akt and ERK pathways when stimulated with insulin (Supplementary Fig. 1b; ref. 5). S2R+ cells expressing yellow fluorescent protein (YFP)-tagged Rolled were seeded in 384-well plates aliquoted with a genome-wide dsRNA collection targeting 20,420 predicted unique genes. Following knockdown, unstimulated or insulin-stimulated cells were stained with fluorescently-conjugated dpERK antibodies, and dpERK fluorescence was converted to ERK–YFP normalized Z-scores (see Methods). In assay validation tests, RNAi of known pathway components such as *PTP-ER*, *InR*, *Ras1* and *Ksr* had expected effects on ERK activation (Supplementary Fig. 1d).

We isolated 1,168 annotated genes (Supplementary Table 1 and Supplementary Fig. 2a, c), including the core RTK/ERK pathway in *Drosophila* (Supplementary Figs 1a, 2d). The assay significantly enriches for genes conserved in metazoans; 62% of the genes have a human orthologue, and 23% have a potential homologue implicated in disease (Supplementary Fig. 2e). A broad array of cellular functions influences ERK signalling output (Fig. 1a), whereas 56% of the hits have no annotated molecular function. We found enrichment for specific gene functions (Supplementary Table 2), including neurogenesis, cytoskeletal maintenance, morphogenesis and cell proliferation. On the basis of median Z-scores, we observed that trafficking genes negatively regulated RTK/ERK activation following stimulation, whereas components of the proteasome and ribosome were positive regulators (Fig. 1b). Finally, we found that 41 genes that regulate both ERK activation and cell morphology⁶ fall into multiple phenotypic categories (Supplementary Table 3), suggesting that the genes control several pathways regulating morphology.

In contrast to the core signalling cassette, other RTK/ERK pathway regulators may be cell-type-specific or required only following stimulus downstream of a particular RTK. For example, in PC12 cells, an NGF-induced, RAP1-mediated, sustained ERK signal leads to differentiation whereas EGF-induced, transient ERK activation leads to proliferation⁷. In *Drosophila*, the requirement for the Shc adaptor is restricted to particular RTKs (ref. 8). We classified our primary hits by secondary screening in two cell lines (S2R+ and Kc167) and under stimulation of distinct RTKs: InR, *Drosophila* EGFR (DER) and the PDGF/VEGF homologue receptor (PVR), which is also active at baseline, stimulated by insulin, secreted Spitz (sSpi) and PDGF- and VEGF-related factor isoforms (PVFs), respectively. Of 362 genes tested, 91% (331) had a significant effect under one or more stimuli (Fig. 2a; Supplementary Tables 4, 5). Because two-thirds of these have mammalian orthologues (Supplementary Table 6), many may also regulate ERK signalling in humans. In separate tests using non-overlapping dsRNAs, we found little contribution to the false-positive rate of off-target effects, probably owing to the filtering of our primary hit list for selection of dsRNAs without significant predicted off-targets (see Methods).

To categorize the activity of 331 genes across seven experimental conditions, we performed hierarchical phenotypic clustering (Fig. 2a). Genes that strongly affected ERK signalling regardless of cell line or RTK-activation (in the same way as known pathway components; Fig. 2b) may be novel components of the core signalling cassette or general permissive factors for ERK activation, such as *CG31302* (which encodes the orthologue of human RIM-binding protein 1/2) and *CG30387* (whose human orthologous protein ARMS is required for sustained ERK activation through Trk; ref. 9), suggesting that some of the identified genes have a broader role in RTK signalling. The *Argonaute1* (*AGO1*) knockdown may prevent biogenesis of miRNAs required for negative regulation of ERK signalling, such as *let-7* family

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members¹⁰. Importantly, CG30476 was recently discovered as a novel Cnk interactor modulating RTK activity during *Drosophila* development¹¹, illustrating the power of our approach to discover new, core signalling pathway components.

Other genes modulating ERK activation may be required only in specific contexts (Fig. 2b). InR had little effect under EGF stimulus, whereas PVR unexpectedly potentiated InR signalling but had possibly antagonistic effects after DER activation. Similar RTK specificity was observed for the conserved genes *Serotonin transporter* (*SerT*), *zinc finger homeodomain 1* (*zfh1*) and CG3878, the orthologue for which was isolated from a screen in mammalian cells for tumour suppressors¹². We also observed cell-line specificity for regulators such as CG6701, a putative transcription factor/helicase, and *Ras64B*, a Ras paralogue, potentially as a result of the relative expression in each cell line.

Particularly striking in cell-type specificity were components of the cell cycle machinery. Whereas cell cycle progression is a consequence of ERK activation^{13,14}, this effect can also be cell-type dependent¹³. Our results suggesting that cell cycle components constitute a feedback mechanism for regulating ERK signalling is consistent with cdc2-mediated Mos accumulation activating MAP kinase (MAPK) in *Xenopus*¹⁵. As has been shown for the Jak/STAT pathway in *Drosophila*¹⁶, cell cycle machinery regulating signal transduction may be direct rather than through cell cycle control.

We isolated many other genes that have been characterized as ERK effectors, such as components of the general transcription machinery¹⁷ (Supplementary Table 4). This is consistent with the synthetic multivulva (*synMuv*) negative regulators of vulva formation in *Caenorhabditis elegans*¹⁸ and isolation of general transcription factors from multiple *Drosophila* genetic screens for RTK/ERK regulators. More broadly, we found 82 genes that also seemed to be regulated by the pathway, as determined from two recent transcriptional profiling studies *in vivo*^{14,19} (Supplementary Table 7). Our results demonstrate that steady-state, feedback inhibition or amplification of ERK signalling following component depletion may be more extensive than is currently appreciated from studies of single genes. Just as the targets of RTK/ERK signalling are diverse, this pathway integrates a similar diversity of cellular processes in determining the precise level of output.

Signal specificity can arise by cross-talk with other pathways within the signalling network or at transcriptional targets. Although individual components of other canonical pathways were identified (for example, *cact*, *shn*, *fz2*), the JNK/MAPK and Akt/Tor pathways consistently negatively affected RTK/ERK signalling (Supplementary Fig. 4b, c). As AP-1 transcription factors *D-jun* (*Jra*) and *D-fos* (*kay*) are direct effectors of the JNK and ERK MAPKs¹⁷, their isolation as negative regulators suggests feedback on proximal pathway activation. Similarly, positive regulators of the Akt/Tor pathway, such as *Akt*, *Rheb* and *Tor*, negatively regulated ERK, whereas negative regulators such as *Pten* and *Tsc2* (also known as *gig*) positively regulated ERK activation. This is consistent with the recent demonstration that mammalian *Akt1* knockdown induces ERK activation²⁰ and Akt inhibits Raf through phosphorylation²¹. Although Akt phosphorylation sites in *Drosophila* Raf are conserved, the direction of ERK regulation by pathway components downstream of Akt is more consistent with negative feedback within the Akt pathway at the level of InR itself²², and, hence, this is possibly how Akt affects the ERK pathway.

Although mutations in or overexpression of multiple RTKs have been implicated in various cancers¹, 30% of solid tumours have mutations in Ras or Raf (ref. 2). To determine if the novel regulators isolated following RTK stimulus can also modulate ERK activation downstream of oncogenic Ras signalling (Ras^{V12}), we screened our validated hits in a stable S2 cell line conditionally expressing Ras^{V12} (ref. 23) (Fig. 2c). We found that 85 genes suppress ERK activation following Ras^{V12} expression (Supplementary Table 8, 9). In parallel experiments, ectopic Ras activity was induced by pre-incubation with dsRNA targeting a *Drosophila* RasGAP, *Gap1*, mimicking loss of NF1 activity in patients with neurofibromatosis, type 1 (Supplementary Table 9). These epistasis analyses suggest where, within the canonical pathway, these genes modulated ERK activation; genes that reduce ERK activation by insulin but not by Ras^{V12} may function upstream of Ras or in a parallel InR–ERK pathway.

Genes influencing RTK/ERK signalling output in cell culture and *in vivo* are more likely to be critical components of the regulatory network, and thus relevant to mammalian development and disease. Through a series of *in vivo* experiments testing known alleles or transgenic RNAi and complementary DNA overexpression constructs

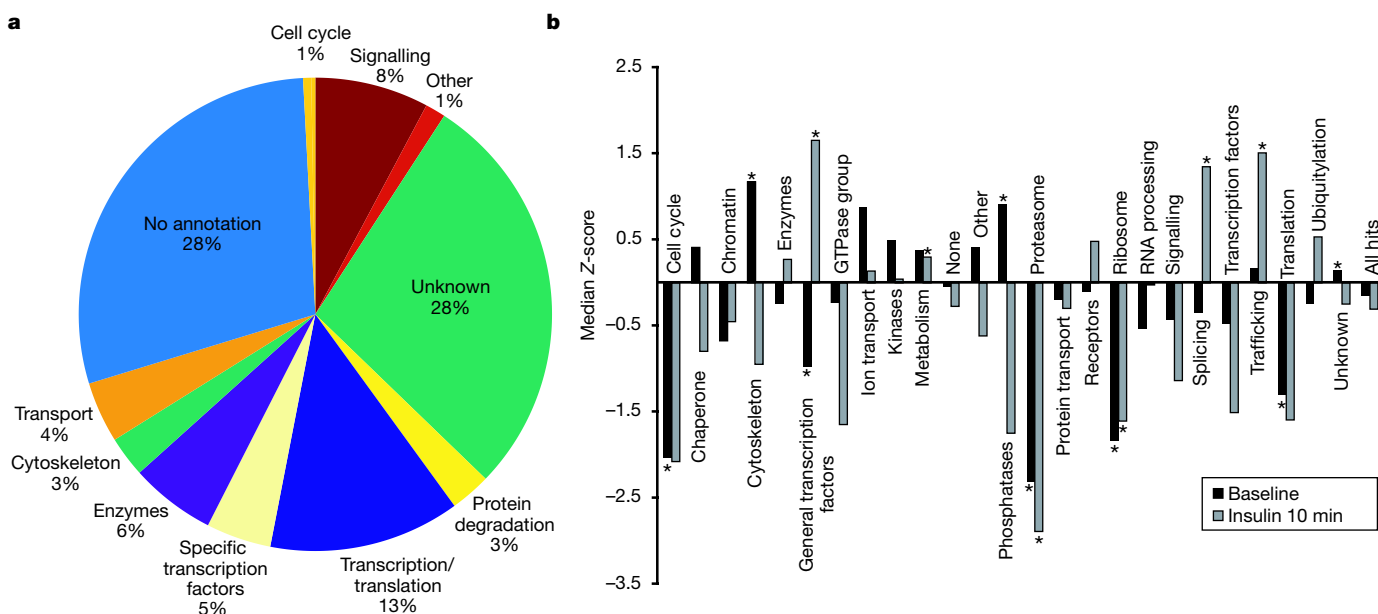


Figure 1 | Primary RTK/ERK RNAi screen results. **a**, Using gene ontology annotations, primary screen hits were grouped by general molecular function. 'No annotation' refers to predicted genes (see Methods). Isolation

of 476 predicted genes suggests that these may be valid transcripts. **b**, Median Z-scores for each functional category in the assay. Asterisk denotes $P < 0.05$.

(not shown), we focused our investigation on two uncharacterized genes, a novel positive regulator and a negative regulator of RTK/ERK signalling.

CG5169/Drosophila GCKIII (dGCKIII) is a member of the Ste20-like kinase group and germinal centre kinase (GCK)-III subfamily. Ste20-like kinases mediate a wide array of signalling events controlling apoptosis, proliferation and cytoskeletal organization²⁴. A *dGCKIII* knockdown reduced ERK activation in stimulated S2R+ cells (Supplementary Fig. 5a). Ras activity in the *Drosophila* imaginal disc is required for survival, proliferation and differentiation, each requiring a specific threshold of ERK activity²⁵. Wing-specific expression of a *dGCKIII* transgenic RNAi hairpin resulted in loss of tissue that was sensitive to hairpin dosage, temperature and gene dosage (Fig. 3a). This phenotype correlated with a consistent reduction in general dpERK staining (Supplementary Fig. 5d). Co-expression of

full-length *dGCKIII* cDNA, but not a kinase-dead form, rescued this phenotype (Fig. 3a), arguing that GCK-III kinase activity is required for its function.

The *dGCKIII* knockdown moderately suppressed ectopic wing veins caused by the EGFR gain-of-function (*Elp^{B1}*) background, and overexpression of *dGCKIII* in flies sensitized by heterozygosity for *Gap1* produced ectopic wing veins, suggesting that *dGCKIII* regulates ERK signalling *in vivo* (Fig. 3b and Supplementary Fig. 5e). Because cell survival (Fig. 3c), but not wing vein differentiation, seems to be the primary phenotype of reduction of *dGCKIII* gene dosage, dGCKIII may be required only for low-level survival ERK signalling or may function in parallel survival pathways. As other Ste20 group members have been characterized as MAP4Ks, we established by co-immunoprecipitation that dGCKIII could bind Raf and protein phosphatase 2A (PP2A) in S2R+ cells (Fig. 3d). As dGCKIII

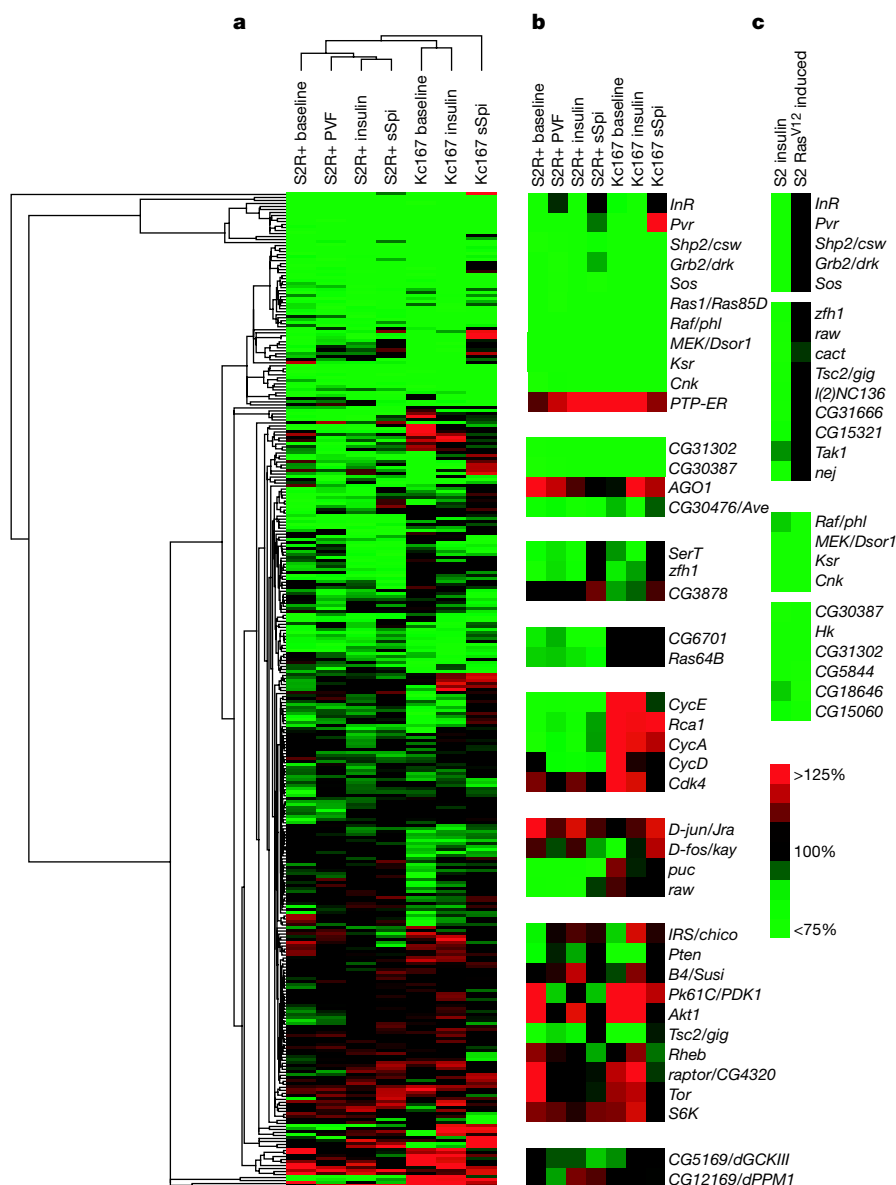


Figure 2 | Secondary screening of RTK/ERK regulators. **a**, Hierarchical clustering of hits validated in two cell lines and under multiple RTK stimuli (insulin, sSpi and PVF). Values are dpERK intensities normalized by total ERK (see Methods). Scale is per cent of a luciferase negative control; negative regulators are indicated in red and positive regulators in green. Preferential clustering of secondary screens by cell line rather than ligand stimulus suggested a large contribution of cell-line specificity to our phenotypic variability. The same clustering with associated gene names is

provided in Supplementary Fig. 4a. **b**, Selected groups of genes that are described in the text. **c**, Selected genes screened in an S2 cell line conditionally expressing Ras^{V12}, under insulin stimulus or following 24 h Ras^{V12} induction. dsRNAs targeting genes that suppress ERK activation following insulin stimulus, but not Ras^{V12} stimulus (as is the case for known upstream canonical components) may function upstream of Ras in the RTK/ERK pathway. Also shown are examples of genes that suppress dpERK following both RTK and Ras^{V12} stimuli.

appears to be membrane-localized (Supplementary Fig. 5g), dGCKIII may be a component of the Raf activation complex containing PP2A²⁶. Finally, siRNA-mediated knockdown of dGCKIII orthologues *MST3* and *MST4* in human prostate cancer cells reduced basal ERK1/2 activation (Supplementary Fig. 5h). As *MST4* expression is elevated in

poorly differentiated prostate cancer cells, and a kinase-dead mutant can inhibit *in vivo* tumour growth²⁷, our results suggest that GCK-III Ste20 family members may contribute to prostate cancer progression through RTK/ERK signalling.

CG12169/dPPM1 is a member of the *Drosophila* class of highly conserved PPM Ser/Thr phosphatases²⁸. Recently, a PPM family member *alphabet/CG1906* was demonstrated to regulate Ras signalling *in vivo*²⁸. A *Drosophila* PPM1 (*dPPM1*) knockdown increased dpERK in S2R+ cells after insulin activation (Supplementary Fig. 6a). Conversely, overexpression of *dPPM1* strongly suppressed ERK activation; a catalytically inactive *PPM1* failed to suppress ERK activation, but, rather, elevated dpERK levels (Fig. 4a), perhaps acting as a dominant negative.

Wing-specific overexpression of *dPPM1*, but not a phosphatase-dead form, reduced wing size and suppressed the ectopic wing vein phenotype of *Elp^{B1}* (Fig. 4b and Supplementary Fig. 6b). Overexpression also moderately suppressed the rough eye phenotype induced by Ras^{V12} under the control of the *sevenless* promoter (Fig. 4c), arguing that PPM1 reduces RTK/ERK signalling *in vivo* in a catalytically dependent manner. PPM proteins seem to be general 'activation T-loop' Thr phosphatases of substrates such as MAPKs^{29,30}. Consistent with this, we detected dPPM1 and ERK interaction by co-immunoprecipitation (Fig. 4d). The D228A mutant demonstrated increased ERK interaction, as described for a human orthologue with p38³⁰. Finally, siRNA-mediated reduction of the human orthologue PPM1 α in human prostate cancer cells increased ERK activation following EGF stimulation (Supplementary Fig. 6e). This suggests that a role of dPPM1, in particular, and PPM-family phosphatases, in general, as negative regulators of ERK activation is conserved.

In summary, a functional genomic screen for novel regulators of RTK/ERK signalling demonstrates that the precise level of ERK activation is the result of integration of other canonical pathways, a

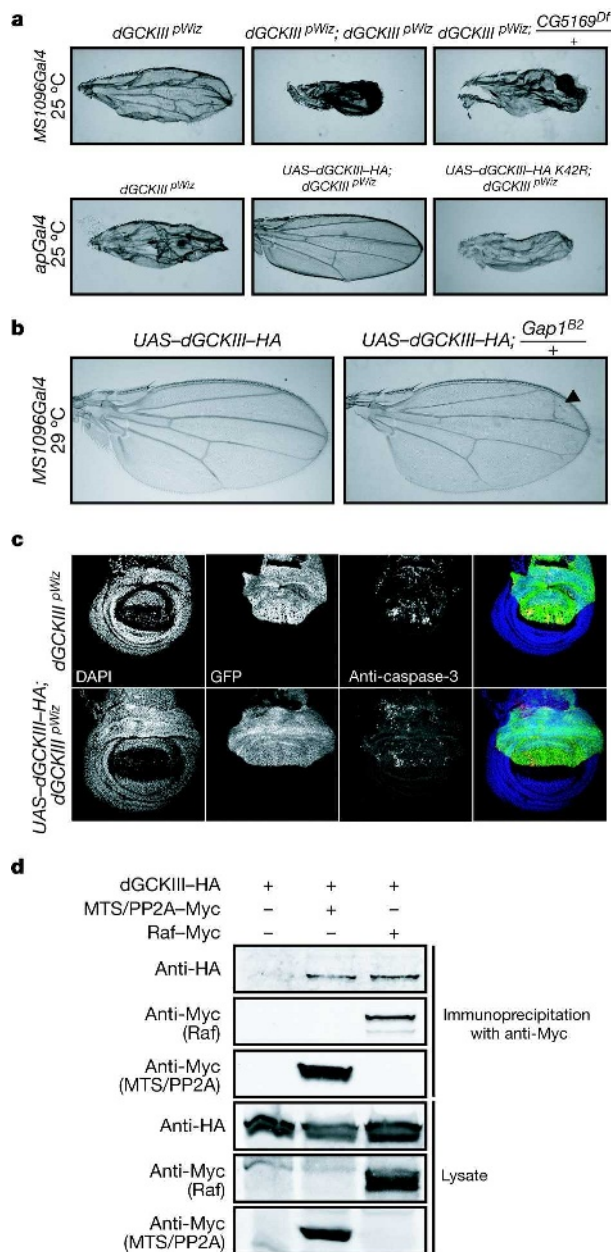


Figure 3 | Characterization of dGCKIII, a novel Ste20-like kinase. **a**, Wing-specific expression of a hairpin targeting *dGCKIII* (*dGCKIII^{pWiz}*), driven by either *MS1096Gal4* or *apGal4* as indicated. The hairpin reduced expression of HA-tagged dGCKIII in wing discs (Supplementary Fig. 5c). *dGCKIII* is ubiquitously expressed in the wing disc (Supplementary Fig. 5b). Tissue loss was increased by doubling hairpin dosage or removing one wild-type gene copy using a deficiency disrupting this gene (*CG5169^{Df}*). This phenotype could be rescued by *dGCKIII* cDNA (*UAS-dGCKIII-HA*), but not a kinase-domain-mutant (*UAS-dGCKIII-HA K42R*) dGCKIII, expressed to similar levels (Supplementary Fig. 5c). **b**, Overexpression of dGCKIII-HA in wing discs. We observed no phenotype of *Gap1B2⁺* or dGCKIII-HA alone (not shown). **c**, Increased cell death, detected with cleaved caspase 3, in wing discs expressing the *dGCKIII* hairpin driven by *apGal4*, marked using *UAS-mCD8-GFP*. Cell death was partially rescuable by co-expression of *dGCKIII* cDNA. No decrease in proliferation was seen (Supplementary Fig. 5f). **d**, Co-immunoprecipitation of dGCKIII by MTS/PP2A and Raf. Interaction with PP2A was predicted from yeast data sets (see Methods).

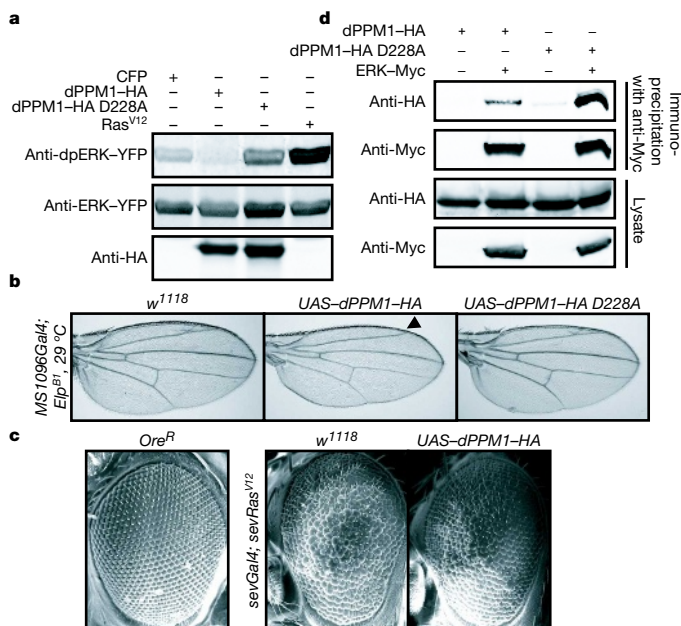


Figure 4 | Characterization of dPPM1, a novel PPM phosphatase. **a**, Overexpression of dPPM1-HA or catalytically inactive dPPM1-HA D228A with YFP-tagged ERK in S2R+ cells, following insulin stimulation. CFP and Ras^{V12} are negative and positive controls, respectively. We found an average 64% decrease in normalized dpERK signal intensity; the D228A mutant elevated dpERK levels by 31% on average. **b**, Suppression of the *Elp^{B1}* ectopic wing vein phenotype by dPPM1, but not the D228A mutant, expressed at similar levels (Supplementary Fig. 6c). **c**, Suppression of the *sevenless-Ras^{V12}* rough eye phenotype by dPPM1. *Ore^R*, wild type; *w¹¹¹⁸*, negative control. **d**, Co-immunoprecipitation of dPPM1 and dPPM1 D228A by ERK. The D228A mutant protein was consistently more efficiently precipitated with ERK than the wild-type protein.

wide range of biological processes, and extensive pathway feedback. Integrating this data with other genome-wide approaches should provide a more complete understanding of the cellular response to RTK signals. As targeted cancer therapies—that inhibit the growth of malignant cells by interfering with the causative mutant signalling pathways—begin to supplement conventional cytotoxic chemotherapy, the need to identify novel and specific drug targets in these pathways is becoming more urgent. Pathway-specific functional approaches complement phenotypic-driven screens in mammalian systems¹², both of which may lead to the identification of novel targets for drug discovery.

METHODS

The genome-wide dsRNA screening collection of the *Drosophila* RNAi Screening Center covering >95% of the *Drosophila* genome has been previously described. We observed similar kinetics of ERK activation and effects of RNAi of known pathway components in the Rolled–YFP stable S2R+ cell line compared to wild-type cells (see Supplementary Figs 1–3). Furthermore, all secondary screens were performed in wild-type cells. We adapted previous antibody-based assays⁶ for our high-throughput screen. Monoclonal dpERK antibodies (Cell Signaling) were directly conjugated to Alexa 647 dye using the Alexa Fluor 647 Protein Labeling Kit (Molecular Probes). Each time point (baseline and stimulated) was screened in duplicate.

For secondary screening, we used both total ERK and dpERK antibodies (Cell Signaling). Similar plate manipulations were used as in the primary screen, with the addition of secondary antibody incubations. Normalized dpERK values are represented as per cent of controls, and *P*-values were calculated for each gene and assay using the Student's *t*-test. Multiple hypothesis correction was applied using the False Discovery Rate (FDR) criteria ($q < 0.05$ as threshold for all assays). In addition to wild-type cells, two stable cell lines were established (S2R+ and Kc167) that conditionally express DER. Because the primary source of baseline ERK activation is through PVR, genes isolated at baseline but not under insulin stimulus may be specific effectors of PVR activity. We also screened our secondary gene list under hyper-stimulation of PVR (see Supplementary Information for secondary screen assay validation). S2 cells expressing inducible Ras^{V12} were provided by M. Therrien²³. For *Gap1* epistasis, S2R+ cells were pre-incubated with either *luciferase* (*luc*) or *Gap1* dsRNA 48 h before secondary screening.

Standard procedures were used for all other experiments. See Supplementary Information for a more detailed description of the methods.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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