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A genome-wide RNAi screen identifies MASK as a positive regulator of cytokine receptor stability

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Summary statement

A genome-wide RNAi screen, in *Drosophila*, identifies MASK as a positive regulator of the JAK/STAT signalling via stabilisation of the pathway receptor - a function conserved in human cells.

Abstract

Cytokine receptors often act via the Janus Kinase and Signal Transducer and Activator of Transcription (JAK/STAT) pathway to form a signalling cascade essential for processes such as haematopoiesis, immune responses and tissue homeostasis. In order to transduce ligand activation, cytokine receptors must dimerise. However, mechanisms regulating their dimerisation are poorly understood. In order to better understand the processes regulating cytokine receptor levels, activity and dimerisation, we used the highly conserved JAK/STAT pathway in *Drosophila,* which acts via a single receptor, known as Domeless. We have performed a genome-wide RNAi screen in *Drosophila* cells, identifying MASK as a positive regulator of Domeless dimerisation and protein levels. We show that MASK is able to regulate receptor levels and JAK/STAT signalling both *in vitro* and *in vivo*. We also show that the human homologue, ANKHD1, is also able to regulate JAK/STAT signalling and the levels of a subset of pathway receptors in human cells. Taken together, our results identify MASK as a novel regulator of cytokine receptor levels, and suggest functional conservation, which may have implications for human health.

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Introduction

The ability to bind to extracellular ligands and transduce the resulting interaction across the plasma membrane represents the central biological function of cytokine receptors. Such receptors include the single-pass transmembrane proteins that ultimately stimulate the Janus Kinase and Signal Transducer and Activator of Transcription (JAK/STAT) pathway (Arbouzova and Zeidler, 2006, Vainchenker and Constantinescu, 2013). This group of receptors are typically homo- or hetero-dimerised with an extracellular structure consisting of multiple Fibronectin Type III repeats in which two of the distal repeats form a cytokine binding motif (Tanaka et al., 2014) (CBM; Fig 1A). On the C-terminal intracellular side, long, β-type cytokine receptors, such as Glycoprotein 130 (GP130), Oncostatin M Receptor B (OSMRB), Thromobopoietin Receptor (TPOR) and the *Drosophila* receptor Domeless (Dome), contain juxta-membrane domains via which cytosolic JAK tyrosine kinases associate. By contrast, shorter, α-type receptors such as Interleukin (IL)-6Rα, participate in the formation of ligand binding complexes but lack the intracellular domains bound by downstream pathway components (Heinrich et al., 2003).

Cytokine binding to the extracellular domains of a receptor complex induces a conformational change, which either reorients a preformed dimer (Brown et al., 2005, Remy et al., 1999) or induces receptor dimerisation/oligomerisation (Thomas et al., 2011). In the case of Erythropoietin (EPO), ligand binding has been shown to be sufficient to bring about receptor dimerisation (Boger and Goldberg, 2001) while the related receptor in *Drosophila*, Domeless (Dome),

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is dimerised *in vivo* via a ligand and JAK/STAT pathway-independent mechanism (Brown et al., 2003).

In canonical JAK/STAT pathway signalling, this activation results in JAK auto-phosphorylation, followed by trans-phosphorylation of intracellular tyrosine residues in the receptor tail and recruitment of latent STAT molecules (Vainchenker and Constantinescu, 2013). These STATs are then themselves tyrosine phosphorylated, dimerise and translocate to the nucleus where they bind to palindromic DNA sequences in the promoters of pathway target genes and thus regulate gene expression.

In humans, JAK/STAT pathway signalling is mediated by 4 JAKs and 6 STATs, playing key roles both during embryonic development, adult homeostasis and multiple diseases (Arbouzova and Zeidler, 2006, Vainchenker and Constantinescu, 2013). These core components are themselves downstream of multiple receptors and ligands with cell-specific differences, redundancy and cross talk between pathway components making the dissection of signalling processes particularly challenging. For example, signalling by the pro-inflammatory cytokine IL-6, occurs via receptor heterodimers made up of the long β-type GP130 receptor and the shorter α-type IL-6R, with both membrane-bound and soluble forms of IL-6R able to form signalling competent complexes with GP130 to stimulate the downstream pathway (Tanaka et al., 2014). By contrast, the production of erythrocytes and platelets is dependent on homo-dimerised EPO Receptor (EPOR) and TPOR respectively, receptors which function upstream of JAK2 and STAT5 (Seubert et al., 2003).

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Although the core JAK and STAT pathway components have been extensively studied, the regulatory processes controlling upstream pathway receptors are less well understood. One key mechanism regulating receptor levels at the plasma membrane is endocytosis. Originally considered as a mechanism to attenuate pathway signalling following activation (Liu and Shapiro, 2003), it is now clear that the endocytosis and trafficking of ligand:receptor complexes into endosomes, and continued pathway signalling from this internalised compartment, not only occurs, but is also frequently qualitatively changed (reviewed in (Cendrowski et al., 2016). Although uncertainty remains, changes in the micro-environment within a maturing endosome such as reduced pH, trapping of the ligand, alterations in the receptor complex and changes to ligand:receptor affinities are all likely to occur (Kurgonaite et al., 2015). Indeed there is compelling evidence that even closely related receptors, Interferon (IFN) type I and type II, are regulated through varying mechanisms (de Weerd and Nguyen, 2012). Ultimately, receptor recycling to the plasma membrane or destruction of the complex within the lysosome also changes the levels of functional receptors (Chmiest et al., 2016, Gesbert et al., 2004).

In order to better understand the processes regulating cytokine receptor levels, activity and dimerisation, we set out to exploit the lower complexity of the *Drosophila* JAK/STAT signalling pathway which consists of a single JAK and STAT-like molecule together with a single full-length receptor, Dome, and a single short antagonistic receptor, termed Latran [also known as Eye Transformer] (reviewed in (Zeidler and Bausek, 2013). Using this system we undertook an RNA-interference (RNAi)-based screens for regulators of the

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Dome receptor. A previous report indicated that JAK/STAT pathway activation downstream of the Dome receptor requires homo-dimerisation of the receptor (Brown et al., 2003). Furthermore, it showed that dimerisation is developmentally regulated by an as-yet unidentified ligand- and signalling-independent mechanism *in vivo*. In this study, we employed a molecular complementation assay utilising two truncated forms of the β-galactosidase (β-gal) enzyme termed Δα and Δω (Rossi et al., 1997) and fused these to the cytosolic, C-terminal ends of the Dome receptor. Although enzymatically inactive in isolation, dimerisation of two Dome molecules brings together both a Δα and a Δω truncation, allowing molecular complementation and the reconstitution of β-gal activity (Fig. 1A).

Here we present our use of such a molecular complementation assay to undertake RNAi screens for factors able to modulate Dome levels and/or dimerisation. We present our genome-wide analysis of this screen and go on to follow up by analysing the conserved, Multiple Ankyrin repeats and KH-domain containing protein, MASK. Using both biochemical and genetic approaches, we show that MASK promotes Dome dimerisation and stability and demonstrate that JAK/STAT pathway activity is reduced following MASK knockdown. We go on to demonstrate that MASK binds directly to the Dome receptor via its medial A2 cluster of Ankyrin repeats and stabilise the resulting complex. We show that the conserved human homologue, ANKHD1, is also able to regulate both JAK/STAT pathway activity and the stability of a subset of human cytokine receptors. This study therefore identifies a novel regulator of cytokine receptor levels providing insights into the regulation of this disease-relevant signalling pathway.

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Results

A split β-galactosidase assay for monitoring receptor dimerisation

Genome-scale RNAi screening has previously identified multiple regulators of JAK/STAT transcriptional activity (Kallio et al., 2010, Müller et al., 2005). However, changes in gene expression do not provide insights into the molecular mechanisms via which regulators of the pathway act. We therefore modified an assay for Dome dimerisation using a split β-gal complementation system (Brown et al., 2003), in which the coding region for the β-gal enzyme containing one of two inactivating deletions (termed Δα and Δω) was attached to the intracellular terminus of the Dome receptor (Fig. 1A). The Δα and fragments are themselves unable to complement unless they are brought into close proximity by fusing them to proteins that physically interact (Rossi et al., 1997). As previously demonstrated *in vivo* (Brown et al., 2003), each individual β-gal fusion protein is inactive in isolation and shows enzymatic activity only when co-expressed in the same cells (Fig. S1A). Thus we note that all combinations of tagged Dome receptor can dimerise (e.g. DomeΔα :DomeΔα), but homodimers will not be detected as having β-gal activity. However, since all dimer combinations are assumed to form with equal probability, the detectable population of heterodimers are representative of the overall population of all dimerised molecules. We adapted this technique for use in cultured *Drosophila* cells (Fig. 1B) and optimised a luminescent readout for β-gal enzymatic activity (Fig. S1A and Materials and Methods). Although designed to detect receptor dimers, our assay was also inherently

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sensitive to the absolute level of these dimers, since any changes in the amount of protein would also result in changes in β-gal activity.

A genome-wide RNAi screen identifies modulators of Dome dimerisation and stability

We performed a genome-wide RNAi screen using a second generation, *in silico* optimised, double-stranded RNA (dsRNA) library targeting 97.9% of the *Drosophila* genome (Fig. 1C, Fig. S1B) and analysed the resulting >110k data points using best practice analysis techniques (Fisher et al., 2012). To avoid variation in transfection efficiency, which could affect results in the assay, we transfected a single batch of pooled cells that was aliquoted across a whole genome replicate. As expected, negative controls (targeting *GFP* or the *C.elegans* gene *zk686.3*) did not significantly affect our assay while knockdown of the endocytic trafficking component *rab5* increased levels of dimerised Dome, consistent with previous reports (Stec et al., 2013, Vidal et al., 2010). Conversely, knockdown of either *dome* itself or *lacZ* strongly decreased β-gal activity (Fig. 1D; Fig. S1B). Using techniques previously developed for similar genome-scale screens (Fisher et al., 2012, Müller et al., 2005), we first analysed three replicates of initial screening [available via GenomeRNAi (http://www.genomernai.org)] and then identified potential hits, which we subsequently retested in secondary re-screens (Table S1). Based on both primary and secondary screening, 43 candidates with consistent and robust Z-scores were selected for further analysis (Table 1; see Materials & Methods for precise selection criteria). Previous work undertaken *in vivo* suggested that ligand expression is not sufficient for Dome dimerisation

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(Brown et al., 2003). To test this finding in the context of our 43 candidates, we repeated the original Dome dimerisation assay in the presence of co-expressed pathway ligand and found that most (79%, 34/43) of the original hits reproduced their effects (Table 1). In addition, it has also been shown that Dome can form hetero-dimers with the short negatively acting pathway receptor Latran (Lat), and that Lat can also form homodimers with itself (Fisher et al., 2016, Makki et al., 2010). We therefore set up cell based assays to test for Dome:Lat heterodimer and Lat:Lat homodimer formation and used this to test the 43 candidate genes. We found that 90% (36/40) of candidates affect Dome:Lat and Lat:Lat dimers, with 31 of these common to both (Table 1).

Although our molecular complementation assay requires receptor dimerisation to produce β-gal enzymatic activity, changes in signal can also be a consequence of changes in overall receptor levels due to alterations in gene expression level, mRNA stability or protein stability/turnover. In order to distinguish between those hits that promote or inhibit dimerisation and those that simply change protein levels, we next sought to quantify total protein levels using an independent technical approach. We therefore used quantified Western blotting undertaken in triplicate (see Materials and Methods for assay design) to examine the effects of the 43 genes on overall Dome protein levels. Of the candidates tested, 31 altered Dome protein levels by at least 25% (Table 1, also see Fig. S1C-D for examples) while the remaining 13 appear to change dimerisation without affecting overall protein levels. It should be noted that this secondary assay used a different form of tagged Dome and so protein concentration cannot be directly compared to quantitative changes in

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the enzymatic activity of β-gal measured in the original screen (i.e. a 25% change in protein levels would not necessarily relate to a 25% change in luciferase values).

Given the changes in Dome dimerisation and protein levels we also assessed the effect of our hits on JAK/STAT dependent transcription using the *6x2xDrafLuc* reporter (Müller et al., 2005). Surprisingly, while gene knockdown by some dsRNAs clearly affected JAK/STAT transcriptional activity, a large proportion had little or no effect on the *6x2xDrafLuc* reporter (Table 1). This rather unexpected result suggests that either the levels of dimerised receptor are not rate limiting in this system, or that alternative regulatory pathways are able to compensate for changes in Dome dimer activity.

We next undertook an analysis of our 43 candidates to identify gene ontological terms disproportionately enriched or depleted relative to the whole *Drosophila* genome (Mi et al., 2017). This identified strong overrepresentation of genes involved in endocytosis (GO:0006897), actin cytoskeleton (GO:0015629), and cellular component morphogenesis (GO:0032989).

One striking GO group identified were Actin-related proteins initially identified as strong hits, a group of hits which also resulted in significant up-regulation of Dome protein levels (Table 1 and Fig. S1E). Upon further examination by qPCR, we found that RNAi-mediated knockdown of *Act42A* resulted in a significant increase in the transcription of Dome construct transfected into our cells and expressed by an *actin5c*-derived promoter (Fig. S1F). Given that this result indicated the existence of a feedback loop regulating the Actin promoter

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used in this construct, Actin-related genes were classified as non-specific in this assay.

MASK regulates levels of dimerised Dome

Throughout the multiple rounds of screening and secondary assays undertaken, RNAi targeting MASK consistently generated strong effects on the dimerisation assay, receptor levels and JAK/STAT pathway transcriptional activity (bolded in Table 1). We therefore, set out to better investigate the mechanisms underlying this activity.

In order to confirm the screen-based identification of MASK, we retested its effect using an alternative dimerisation assay. Using co-immunoprecipitation of differentially epitope tagged Dome molecules followed by quantification of western blots, we found that knockdown of MASK was sufficient to reduce Dome dimerisation by 50% (± 10%, $p<0.013$, $n=3$) (Fig. 2A). Furthermore, given the nature of immunoprecipitation experiments, this approach is independent of potential protein level changes and therefore represents a specific assay for Dome:Dome dimerisation. In order to assess whether MASK knockdown also altered Dome-FLAG protein levels, we returned to our semi-quantitative western blotting secondary assay. This allowed us to confirm that change in Dome dimerisation, due to MASK knockdown, was also accompanied by an approximate 25% decrease in the steady state level of Dome protein detected (Fig. 2B). As such, knockdown of MASK resulted in both the destabilisation of Dome:Dome dimers and also a reduction in the absolute levels of Dome itself.

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Consistent with the decrease in levels and dimerisation of receptor, we also found that Upd-induced JAK/STAT transcriptional activation was reduced following knockdown of MASK, both at the level of a luciferase-based JAK/STAT-sensitive reporter (Table1) and also via the reduction in transcription of the STAT92E target gene, *SOCS36E* (Fig. 2C). This result was confirmed using two independent dsRNAs, each of which reduced both MASK mRNA by >70% (Fig. S2A), and pathway-induced transcription (Fig. S2B). The requirement for MASK in JAK/STAT signalling was further demonstrated using two additional independent STAT92E reporter assays, each of which was strongly and significantly reduced following MASK knockdown (Fig. S2C). Taken together, these findings confirm that MASK functions as a positive regulator of the *Drosophila* JAK/STAT pathway.

Previous reports have identified MASK as a regulator of the Ras/Raf and Hippo/Warts pathways (Sansores-Garcia et al., 2013, Sidor et al., 2013, Smith et al., 2002), we examined the effects of silencing known components of the Ras (*csw, ras85D, ras64B, raf*) and Hippo (*hpo, wts, yki*) pathways in order to identify potential pathway cross-talk with our JAK/STAT pathway assays. Analysed as Z-scores relative to the original genome-wide screen dataset, neither Dome dimerisation, stability (Fig. S2D) nor STAT92E transcriptional activity (Fig. S2E) were significantly affected by knockdown of any of the Ras or Hippo pathway components tested. This suggests that MASK is acting directly on the JAK/STAT pathway.

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MASK regulates JAK/STAT signalling *in vivo*

In order to support the cell and RNAi-based data, we also undertook *in vivo* JAK/STAT pathway assays using previously characterised loss-of-function MASK alleles (Smith et al., 2002). Ectopic JAK/STAT pathway activation is sufficient to drive over-proliferation within the developing eye imaginal disc, a process that is sensitive to downstream JAK/STAT pathway activity (Fig. 2D-E) (Bach et al., 2003, Mukherjee et al., 2006). Using this test, we found that JAK/STAT pathway-induced eye overgrowth was markedly reduced in genetic backgrounds heterozygous for independent MASK loss-of-function alleles (Fig. 2F and Fig. S2F-H).

We next explored whether MASK was required to maintain Dome protein levels *in vivo*. Since existing MASK mutant alleles are embryonic lethal, we induced mitotic clones of either the hypomorphic allele $MASK^{7.29}$ or the null $MASK^{10.22}$ allele in developing wing discs (Fig. 2G-H). In the absence of reliable antibodies against Dome, we ubiquitously expressed epitope-tagged Dome-V5 throughout the wing disc using *tubulin-GAL4* (Fig. 2G). As observed previously (Makki et al, 2010), Dome was found to accumulate in intracellular vesicles, with weak staining observed at the plasma membrane. Although *MASK* mutant clones proliferate poorly and are therefore relatively small, levels of Dome detected in mutant areas are significantly lower than in surrounding wild type tissue (Fig. 2G-I). When Dome levels inside clones (which are identified by their lack of GFP marker expression) were quantified relative to equivalent neighbouring, wild-type regions, a significant reduction in Dome-V5 levels was observed for both *MASK* alleles (Fig. 2H,I). By contrast another single pass transmembrane protein E-cadherin (E-cad), which is not a

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JAK/STAT pathway receptor, is unaffected by loss of MASK. Given that transcription of Dome in this experiment is driven via a uniformly expressed heterologous *tubulin* promoter, we conclude that changes in Dome are a function of reduced protein levels rather than a change in gene expression. These results suggest that MASK acts as a positive regulator of the JAK/STAT pathway *in vivo* in *Drosophila* and is able to modulate pathway receptor levels.

MASK can physically associate with Dome

Given the ability of ankyrin repeats to mediate protein:protein interactions (Bennett and Chen, 2001, Michaely et al., 1999) and given the ability of *Drosophila* MASK to modulate Dome receptor levels, we reasoned that MASK proteins may directly bind to cytokine receptors. We therefore utilised constructs encoding each of the ankyrin repeat domains and the KH domain of MASK (Sansores-Garcia et al., 2013), expressed these in *Drosophila* Kc_{167} cells (Fig. 3A) and tested these for binding to Dome. We found that the MASK-A2 and, more weakly, the MASK-A1 ankyrin repeat domains were able to co-precipitate Dome-FLAG (Fig. 3B). Although no interaction was detected with the MASK-KH region, we are unable to rule out binding due to much lower expression levels of the MASK-KH fragment (Fig. 3B). The interaction with MASK ankyrin repeat domains was found to be reciprocal, with a FLAG-tagged construct containing both ankyrin repeat domains (MASK-A1A2; Fig 3A) being immunoprecipitated with Dome-HA (Fig. 3C).

These results suggest that MASK forms a physical complex with Dome and suggests that this interaction occurs via its ankyrin repeat domains.

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Increased MASK levels leads to raised receptor levels

Given the negative effect of RNAi-mediated MASK knockdown on receptor stability, we tested whether increased levels of MASK fragments might have the opposite effect. In contrast to loss-of-function experiments, we found that expression of the second MASK ankyrin repeat cluster (MASK-A2; Fig. 3A) (Sansores-Garcia et al., 2013) was sufficient to increase the steady state levels of Dome in Kc_{167} cells (Fig. 3D), while over expression of exogenous Dome was also sufficient to reciprocally stabilise MASK-A1A2 (Fig. 3E). These results support the initial finding that MASK is a positive regulator of Dome stability and, when taken together with the physical interactions between Dome and MASK, suggest that Dome:MASK association forms a stabilised protein complex.

Conservation of MASK function in human cells

Since MASK has been evolutionarily conserved between humans and *Drosophila* at the primary sequence level (Fig. 4A), we tested whether its function in modulating JAK/STAT signalling is also conserved. To test the effects of knocking down the closely related ANKHD1 on the human JAK/STAT pathway, we used qPCR in HeLa cells to detect the mRNA of the pathway target gene, *SOCS3* (Murray, 2007). As expected, mRNA levels of *SOCS3* were strongly decreased following silencing of JAK2 and STAT3 while siRNA-mediated silencing of ANKHD1 (whose efficiency is shown in Fig. S3A) was also sufficient to significantly reduce expression (Fig. 4B). Consistent with this, ANKHD1 knockdown was also sufficient to significantly reduce the OSM-

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stimulated phospho-STAT1 and phospho-STAT3 levels, a hallmark of pathway activation (Fig. 4C and Fig. S3B).

Given that knockdown of *Drosophila* MASK led to a reduction in Dome protein levels (Fig. 2B, 2G) we tested cytokine receptor levels in human cells. Strikingly, while knockdown of ANKHD1 had no detectable effect on Leukaemia Inducible Factor Receptor (LIFR), it led to the almost complete loss of the endogenous GP130, the long cytokine receptor central to IL6-class cytokine receptor complexes (Fig. 4D) (Heinrich et al., 2003). Given that the change in GP130 protein level could be the consequence of changes in protein stability, mRNA stability or transcriptional regulation, we tested the ability of ANKHD1 to alter the levels of HA-tagged TPOR and EPOR expressed from a CMV promoter in HeLa cells in the presence or absence of ANKHD1. Both receptors were greatly reduced following treatment with ANKHD1 siRNA (Fig. 4E,F). Since these receptors were expressed from a constitutive promoter, they are unlikely to be affected by changes in transcriptional control, further supporting a model in which ANKHD1 functions at a post-transcriptional level.

Taken together, these results suggest that the human homologue of MASK, ANKHD1, also acts as a positive regulator of JAK/STAT signalling and modulates the levels of a subset of human cytokine receptors.

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Discussion

The development, and maintenance of multicellular life is absolutely dependent on the ability of cells to communicate with one-another – a process that requires transmembrane receptor molecules. In this report we have undertaken a screen to identify the factors involved in the dimerisation and stability of the *Drosophila* receptor associated with JAK/STAT pathway activation. This single pass, trans-membrane receptor, termed Dome, forms homo-dimers in a spatially and temporally restricted manner during embryonic development. This dimerisation is required for downstream signalling, but is unaffected by the presence of the pathway ligand Unpaired (Brown et al., 2003). More recently, a related, but shorter receptor, termed Latran, was identified which acts negatively to down-regulate JAK/STAT pathway signalling (Kallio et al., 2010, Makki et al., 2010). Strikingly, Latran has also been shown to be able to form both homo-dimers and hetero-dimers with Dome (Fisher et al., 2016, Makki et al., 2010) with the formation of signalling-incompetent Dome:Lat heterodimers thought to represent the mechanism of negative regulation (Fisher et al., 2016). However, while the receptors themselves have been characterised, the mechanisms mediating receptor dimerisation required to generate a signalling-competent complex are unknown. As such, the data reported here represents the first comprehensive description of the components required for this process.

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One caveat of the screen design presented is the fact that the β-gal activity measured is influenced by both the efficiency of dimer formation and the stability/levels of Dome protein – although we are able to rule out effects on transcriptional regulation due to the use of a ubiquitous actin promoter. In order to differentiate between these two influences, we undertook secondary screens using semi-quantitative Western blotting to assess protein levels. In this way we differentiated between those genes modulating dimerisation, those modulating protein levels and those that regulate both aspects. Based on this insight, the identification of hits that change β-gal activity and NOT protein levels (e.g. *sec61β* and *CG6106*) suggest that failure to dimerise does not inherently affect protein stability. By contrast, hits such as MASK that change both dimerisation and protein levels may be affecting both processes, although it is also possible that the loss of receptor stability following MASK knockdown may result in the breakdown of existing dimers as a prelude to protein destruction.

While transmembrane proteins destined for insertion into the plasma-membrane are processed via conserved ER and Golgi pathways, it is clear that knockdown of MASK does not globally affect the production and/or trafficking of all membrane spanning proteins. Rather, the requirement for MASK proteins is specific to a subset of transmembrane proteins affecting Dome but not E-cadherin in *Drosophila* (Fig. 2G-I), and GP130, EPOR and TPOR, but not LIFR, in human HeLa cells (Fig. 4D-F). This is particularly interesting in the context of LIFR, since it is known to form a signalling complex with GP130 (Gearing et al., 1991, Gearing et al., 1992). However, it has been shown that addition of ligand is a key factor in inducing LIFR/GP30

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heterodimerisation, suggesting that GP130 may be trafficked independently of LIFR in unstimulated cells (Giese et al., 2005). Indeed, evidence suggests that LIFR and GP130 can be internalised and degraded via different mechanisms (Blanchard et al., 2000).

In order to obtain a mechanistic insight into the function of MASK, we also undertook a structure-function analysis of MASK itself. This showed that MASK and Dome form stable physical interactions as shown by reciprocal co-immunoprecipitation, with this interaction being primarily mediated by the second, central A2 group of ankyrin domains present in MASK (Fig. 3B). Furthermore, we also show that the overexpression of the MASK-A1/A2 region is able to stabilise Dome levels (Fig. 3E), suggesting that MASK:Dome complexes [and possibly MASK:Dome:Dome complexes] may be inherently more stable than Dome alone. Although largely speculative, it is possible that the interactions seen between Dome and both the A1 and A2 regions of MASK (Fig 3B) may point to a model in which one Dome receptor may bind to each Ankyrin domain so promoting the dimerisation and stabilisation of Dome dimers. Although it is formally possible that MASK alters mRNA stability, this physical association with Dome suggests regulation at the protein level. We have previously demonstrated that Dome is constitutively internalised and degraded via the lysosome, but not recycled to a significant degree (Fisher et al., 2016, Stec et al., 2013). One could therefore speculate that association with MASK stabilizes Dome, slowing the degradation process.

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In humans, *ANKHD1* has a paralogue on chromosome 4, named *ANKRD17* (ankyrin repeat domain 17) (Sansores-Garcia et al., 2013, Sidor et al., 2013) with the two proteins sharing 71% identity, with greater sequence similarity in the regions of ankyrin repeats and the KH domain (Poulin et al., 2003). Strikingly, ANKRD17 has been demonstrated to physically interact with receptors involved in the innate immune response, and plays a role in the release of cytokines (Menning and Kufer, 2013) and interferons (Wang et al., 2012). These findings serve to support our own data and suggest that ANKHD1 and ANKRD17 may also be acting to regulate receptor stability and dimerisation in humans.

Taken together, we present the first systematic screen, which we are aware of, to identify the factors responsible for the dimerisation of a JAK/STAT pathway receptor. We characterise one of these hits, MASK, and show that it regulates JAK/STAT pathway activity and forms a complex with the pathway receptor. We show that MASK is required to maintain the stability of Dome protein both *in vivo* and in cells and may well also play a role in receptor dimerisation. Finally, we demonstrate the evolutionary conservation of the MASK homologue ANKHD1 at the sequence and functional levels. As such, this work provides a valuable insight into this aspect of JAK/STAT pathway and highlights a novel level of regulation of this important and disease-relevant pathway.

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Materials and Methods

Cell culture and biochemistry

Drosophila Kc$_{167}$ cells were obtained from the *Drosophila* Genomics Resource Center (DGRC) and maintained according to standard procedures (Fisher et al., 2012). HeLa cells were maintained in DMEM supplemented with 10% serum. All cells are regularly screened for contamination. Plasmid transfections were carried out using Effectene (Qiagen) according to manufacturer's instructions. Reverse transfections with siRNA were carried out using Lipofectamine RNAiMAX (Invitrogen) using 10nM final concentrations of single siRNAs (Dharmacon), targeting ANKHD1 (D-014405-01 or D-014405-02) where comparable results in terms of knockdown efficiency and reduction in JAK/STAT pathway activity were seen for both, or non-targeting siRNA as a control, D-001210-01. Stimulation of mammalian JAK/STAT pathway was carried out using human recombinant oncostatin M, (295-OM-010, R&D systems) at a final concentration of 10ng/ml for 20 minutes. Immunoprecipitation experiments were carried out as previously described (Stec et al., 2013). Proteins were separated on 4-15% TGX SDS-PAGE precast gels (Bio-Rad) and transferred to nitrocellulose membranes.

Drosophila RNAi screen hits were assessed for their effects on Dome protein levels – although it should be noted that this assay could not distinguish between modulation of mRNA stability or protein turnover. Kc$_{167}$ cells were batch transfected with Dome-FLAG, incubated for 24h, then split into 24-well plates with 4□g dsRNA. After 5d RNAi treatment, cells were lysed as

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described. Lysates were boiled in 2x Laemmeli sample buffer and analysed by western blotting. FLAG/tubulin fold-changes were calculated for each RNAi condition in comparison to the average of three negative controls per gel. Each screen hit was analysed blind in duplicate.

Genome-wide RNAi screening

The genome-wide SRSFv1 library, in 384-well format, was used as previously described (Fisher et al., 2012). Controls were manually added into empty wells (250ng dsRNA in 5ul water): *GFP* and the *C.elegans* gene baring no sequence homology in *Drosophila*, *zk686.3,* were used as baseline controls; technical controls targeting transfected plasmids were *dome, LacZ* and *RLuc*, and *Rab5* was used as a positive control. Genome-wide screening was carried out in biologically independent triplicates. Kc_{167} cells were batch-transfected in T75 flasks with 4μg *pAc-Dome-LacZ-Δ*α*, pAc-Dome-LacZ-Δ*$\underline{\omega}$ and *pAc-RLuc* and incubated for 24h. Cells were pooled in serum-free media, and 15,000 cells seeded per 384-well. After 1h, media was added to a final 10% serum concentration. After 5d cells were assayed for β-gal activity using β-glo Assay System (Promega), which involves a Firefly luciferase reactions (FL), followed by measurement of Renilla luciferase (RL) activity as a viability control. Luciferase activities were measured on a Varioskan plate reader (Thermo). Raw data will be made available on request.

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Data analysis

Firefly and Renilla luciferase values for each well were processed using the CellHTS2 Bioconductor package (Boutros et al., 2006). Values were median centred to normalise for plate-to-plate variation. Ratios of luciferase (FL/RL) were used to calculate the robust Z-scores, which were considered significant ≥2.5 or ≤-2.5. Individual FL and RL values were also assessed, since they were not always linear with respect to one another. Secondary analyses were carried out with newly synthesised dsRNAs and hits were considered significant at the less stringent ≥+2 or ≤-2. Forty-three robust hits were selected at this stage and sequenced to confirm correct target genes.

Drosophila genotypes

Figure 2

D) *w, GMR-updΔ3' / w*^{1118}

E) *w, GMR-updΔ3' / + ;; stat92E*^{397}*/+*

F) *w, GMR-updΔ3' / + ;; MASK*$^{10.22}$*/+*

G,H) *w UbxFLP ;;UAS-Dome-V5 FRT82 MASK*$^{7.29}$ */ tub-GAL4 FRT82 Ubq-GFP*

I) *w UbxFLP ;;UAS-Dome-V5 FRT82 MASK*$^{10.22}$ */ tub-GAL4 FRT82 Ubq-GFP*

MASK alleles were a gift of M Simon (Smith et al., 2002).

Drosophila phenotypes

Eye overgrowth assays were double blind scored alongside *stat92E* and *w*^{1118} out-crosses (n>20 flies per genotype with >2 repeats). Adult flies were photographed using a Nikon SMZ1500 stereo-microscope and Nikon Elements extended depth of focus software package.

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Wing discs were dissected from wandering 3rd instar larvae raised at 25 °C. Inverted carcasses were fixed in 4% paraformaldehyde in PBS for 20min, blocked and incubated in primary antibodies overnight at 4°C. Tissues were washed in PBS containing 0.2% Triton X-100 (PBST) and incubated in secondary antibodies overnight at 4°C. After washing, discs were mounted in mounting media and imaged on Nikon A1R GaAsP confocal microscope using a 60x NA1.4 apochromatic lens, with a pixel size of 70 nm, and the pinhole was set to 1.2 AU.

Antibodies

For western blotting all primary antibodies were used at 1:1000 dilutions: ANKHD1 (Sigma, HPA008718), β-actin (Abcam, ab8226), GP130 (Cell Signaling Technologies, 3732), pSTAT1 (Cell Signaling Technologies, 9167), STAT3 (Cell Signaling Technologies, 12640), pSTAT3 (Cell Signaling Technologies, 9145), FLAG (M2, Sigma), HA (3F10, Roche), *Drosophila* α-tubulin (DM1A, Sigma). For immunohistochemistry, primary antibodies were E-cadherin (dCAD2, DSHB, 1:20) and V5 (E10/V4RR, Invitrogen, 1:500).

Cloning of expression constructs

Dome-LacZ-Δα and -Δω fragments were cut from pUAST vectors (Brown et al., 2003) and ligated into pAc5.1 vector (Invitrogen) using KpnI and XbaI restriction sites (partial digestion of KpnI sites used for Δω). pAc-Dome-FALG and pAc-Dome-HA were described in (Stec et al., 2013). *Drosophila* MASK-A1/A2 was PCR amplified from cDNA clone LD31446 (DGRC). Gateway cloning of PCR product was carried out using the pENTR/D-TOPO Cloning Kit (Invitrogen) and introduced into the pAWF vector (*Drosophila* Gateway Vector

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Collection) using Gateway LR Clonase II Enzyme Mix (Invitrogen), according to the manufacturer's instructions. HA-MASK constructs were a gift from G Halder (Sansores-Garcia et al., 2013).

Quantitative real-time PCR

Total RNA was extracted from cells using TRIZOL Reagent (Invitrogen) following manufacturer's instructions. Synthesis of cDNA was carried out using High Capacity RNA-to-cDNA Kit (Applied Biosystems) from 2 □g total RNA. To confirm gene knockdown by RNAi or to measure levels of target gene expression, qPCR was carried out using SsoAdvanced SYBRGreen Supermix (BioRad) on a CFX-96 Touch new generation Real-Time PCR Detection System (BioRad). Change in expression levels between experimental conditions was calculated compared to housekeeping gene expression (either *Drosophila* RpL32 or human β-actin) using the $\Delta\Delta C_T$ method (Bina et al., 2010). Statistical analysis was carried out using one-way ANOVA tests in Prism (Graphpad). Primers are listed in Table S2. TAQMAN qPCR probes were designed for multiplexing (IDT oligo).

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Competing interests

No competing interests declared.

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Figures

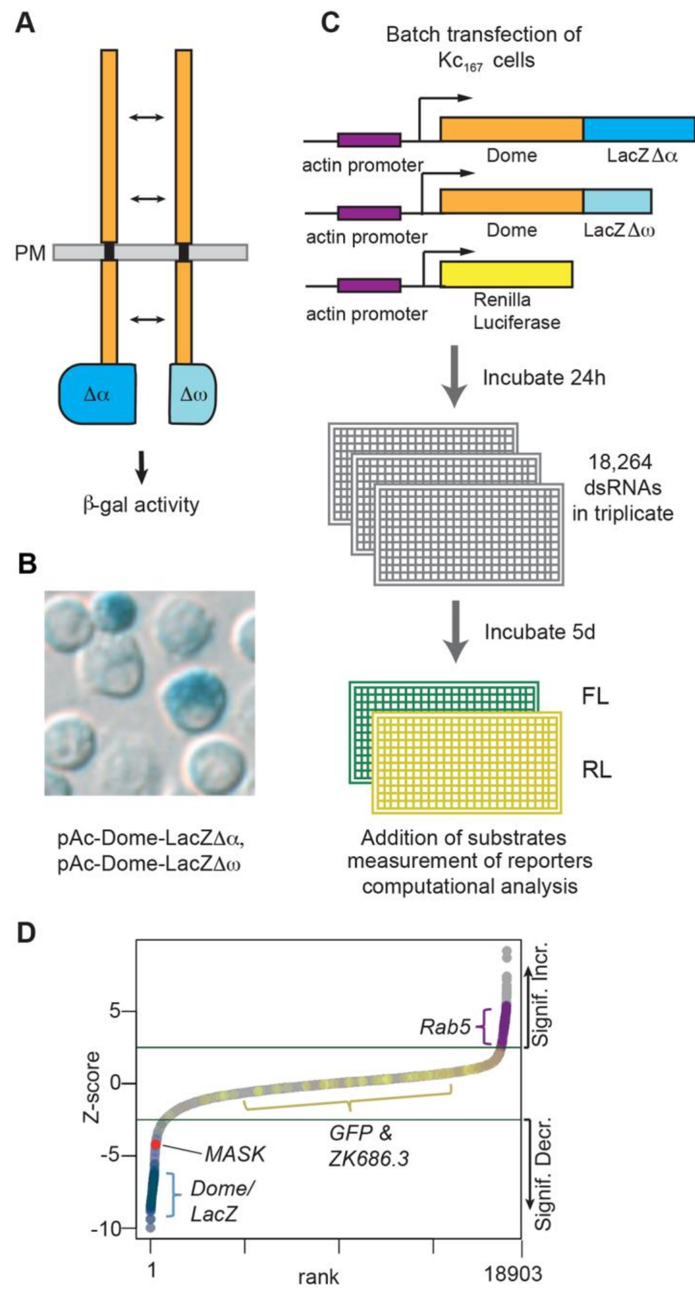

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Figure 1. A split β-galactosidase genome-wide RNAi screen for modulators of Dome dimerisation and levels.

A) Schematic representation of the Dome-βgalΔα and Dome-βgalΔω complementation assay. PM = plasma membrane.

B) *Drosophila* Kc_{167} cells transiently transfected with plasmids expressing the proteins shown in (A) show β-galactosidase activity by X-gal staining.

C) Workflow of the genome-wide RNAi screen for modulators of Dome dimerisation and levels as undertaken in *Drosophila* Kc_{167} cells.

D) Ranked Z-scores from the genome-wide RNAi screen. Green lines illustrate Z-score cut-offs of significant increase or significant decrease. Controls are shown with *MASK* highlighted in red.

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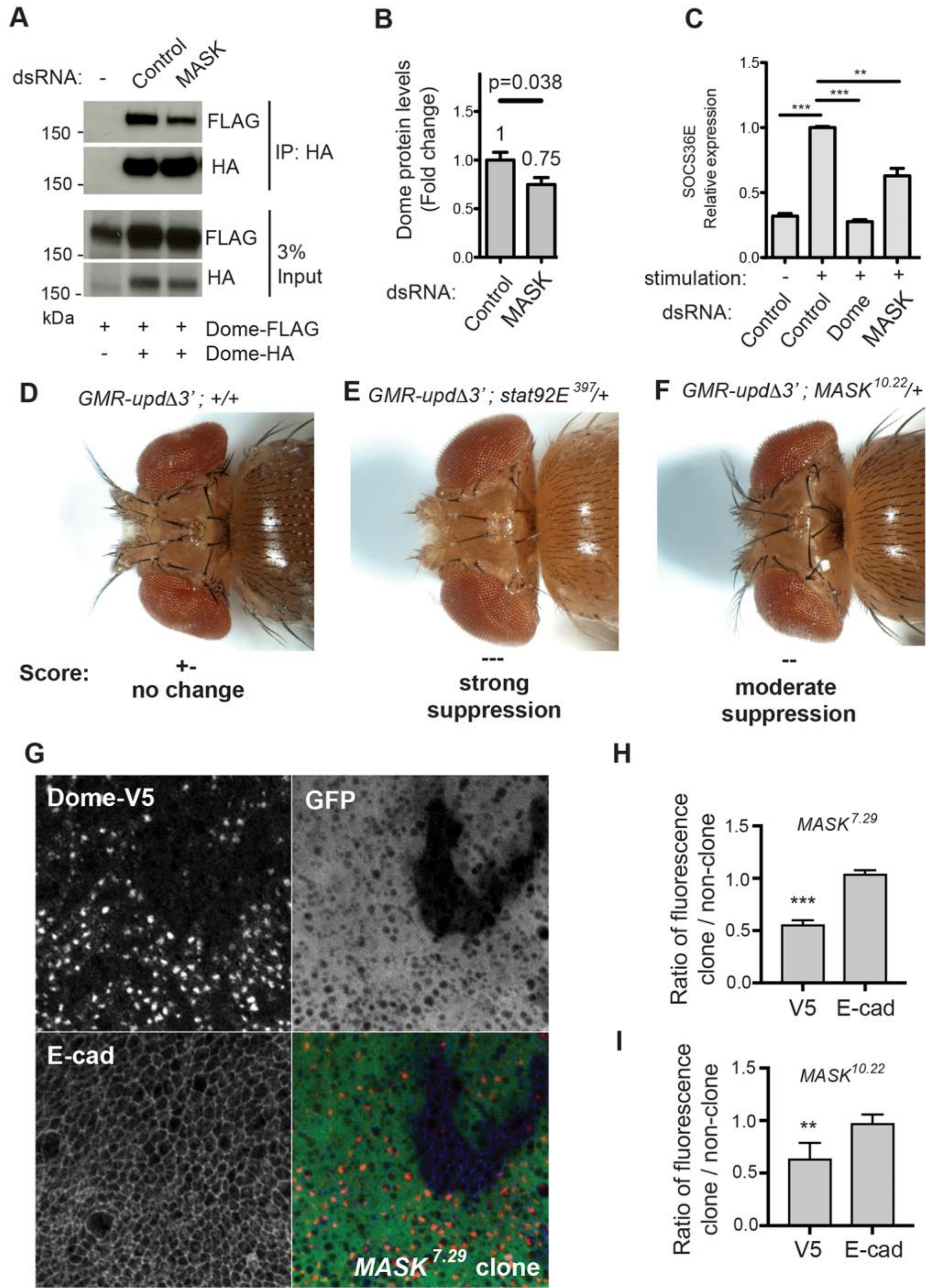

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Figure 2. MASK regulates pathway activity and receptor levels *in vivo*

A) Co-expression of Dome-HA and Dome-FLAG followed by Dome-HA immunoprecipitation in *Drosophila* Kc_{167} cells. Levels of co-precipitated Dome-FLAG are modulated by treatment with the MASK dsRNA.

B) Quantification of steady state Dome-FLAG protein levels expressed by Kc_{167} cells after knockdown of MASK. Number indicates fold change, error bars show standard deviation, p-value is indicated from Student's t-test (n=3).

C) Expression of the JAK/STAT pathway target gene *SOCS36E* following Upd2 ligand stimulation and treatment with indicated dsRNAs. Error bars show standard deviation (n=3), ** $p < 0.01$, *** $p < 0.001$ (One-way ANOVA with Dunnett's posthoc test).

D-F) Dorsal view of eye overgrowth phenotypes caused by ectopic Upd ligand expression driven by *GMR-UpdΔ3'.* Loss of one copy of *STAT92E* or *MASK* suppresses overgrowth.

G) Mitotic clones of $MASK^{7.29}$ caused a reduction in *tubulin-GAL4* driven UAS-Dome-V5 (red) fluorescence, whereas E-cadherin (blue) levels were unaffected. Clones were identified using loss of native GFP (green).

H-I) Quantification of Dome-V5 and E-cad levels in $MASK^{7.29}$ (H) or $MASK^{10.22}$ (**I**) mutant clones. Ratios of fluorescence intensity inside clones and in nearby twin-spots were taken to control for variations across discs. Measurements were averaged over ≥4 discs with at least 2 clones per disc. ** $p < 0.01$, *** $p < 0.001$ (One-sample t-test with expected mean of 1).

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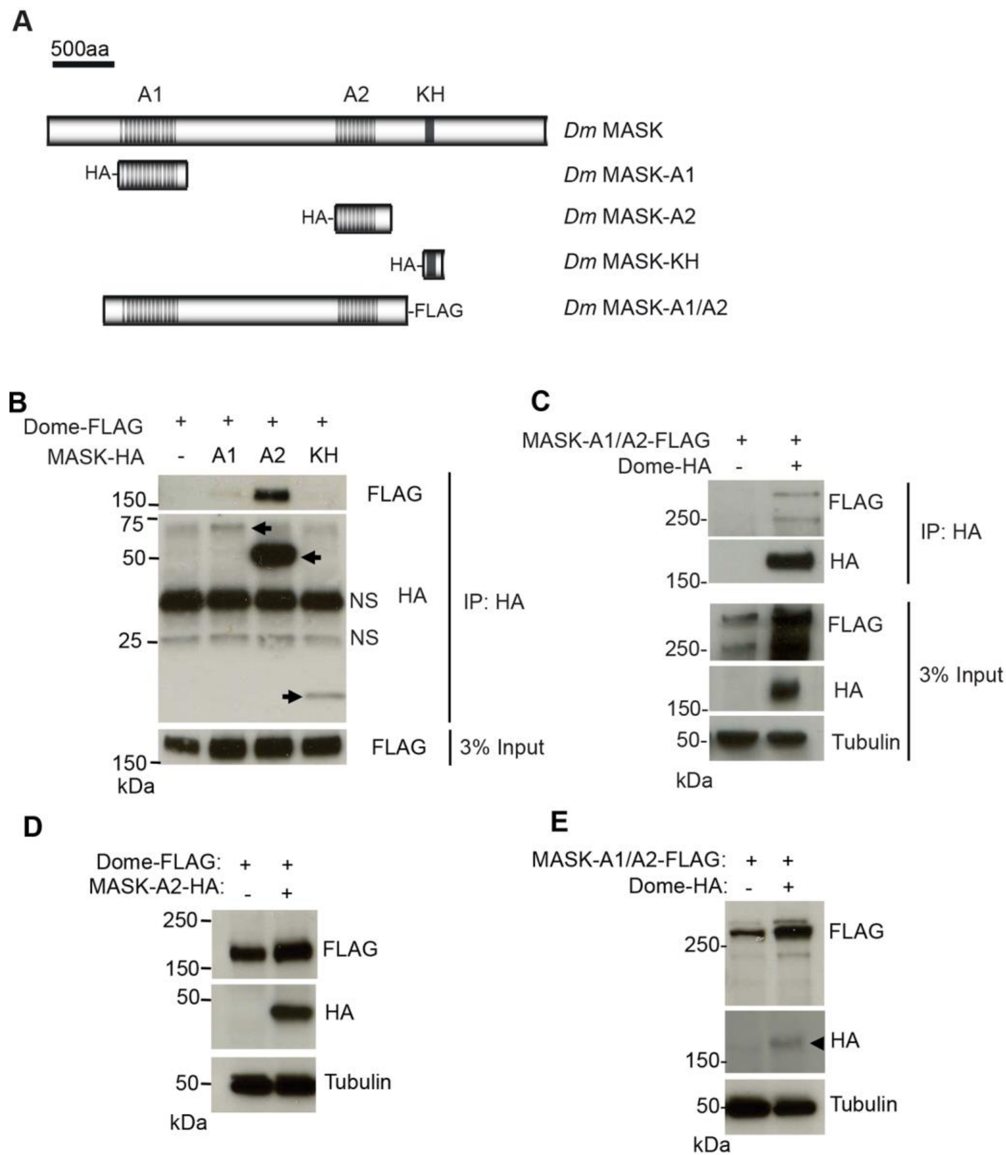

Figure 3. MASK physically associates with Dome.

A) Schematic representation of *Drosophila* MASK protein and constructs used in this study.

B) Immunoprecipitation of the indicated HA-MASK constructs from Kc_{167} cells also expressing Dome-FLAG. Dome-FLAG is co-immunoprecipitated with HA-MASK-A1 and HA-MASK-A2. Levels of Dome-FLAG present in the input lysate are shown. NS = non-specific band.

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C) Co-precipitation of MASK-A1/A2-FLAG following immunoprecipitation of Dome-HA. Levels of MASK-A1/A2-FLAG, Dome-HA and α-Tubulin present in the total Kc_{167} cell lysates are shown.

D) Steady state levels of Dome-FLAG expressed in *Drosophila* Kc_{167} cells are increased following the co-expression of HA-MASK-A2. Levels of α-Tubulin indicate loading parity.

E) Steady state levels of MASK-A1/A2-FLAG expressed in *Drosophila* Kc_{167} cells are increased following the co-expression of Dome-HA. Levels of α-Tubulin indicate loading parity.

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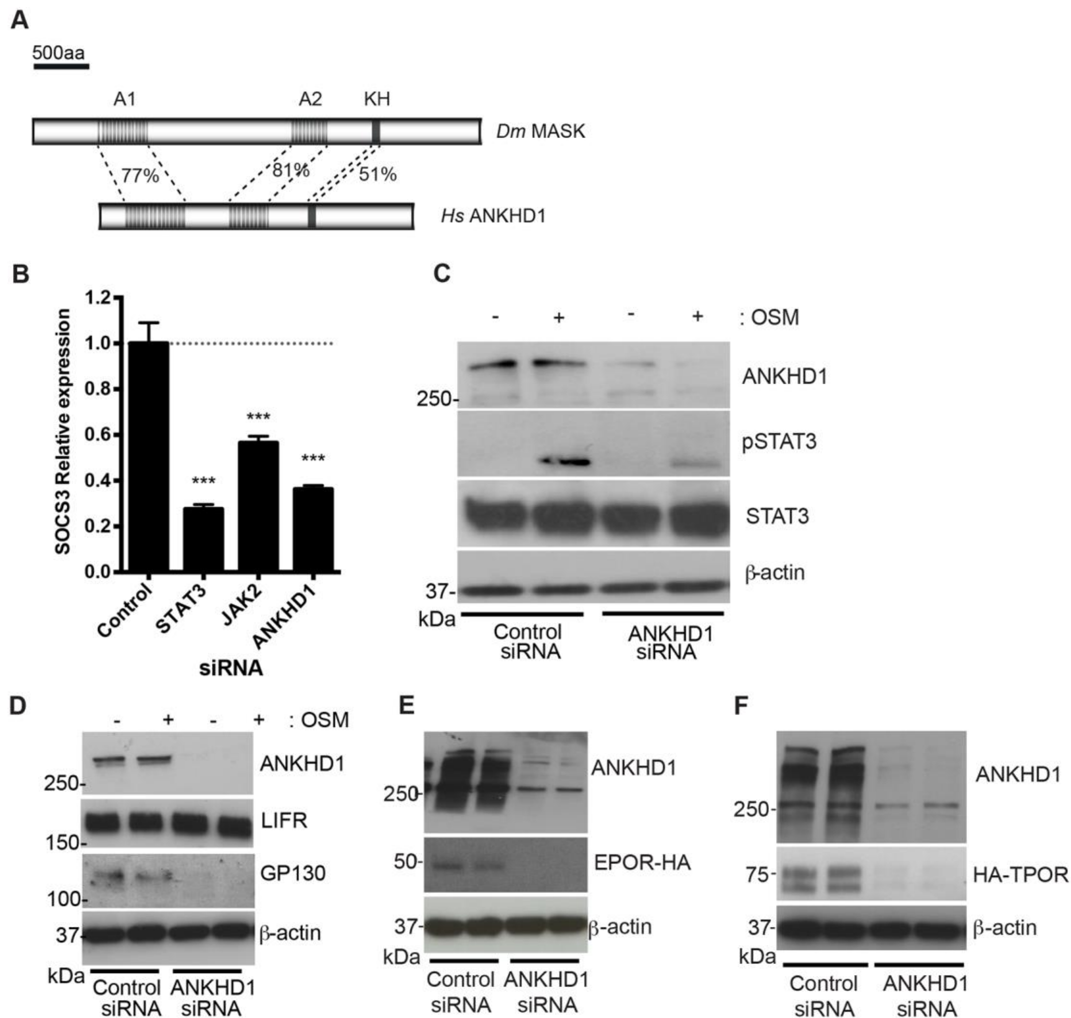

Figure 4. MASK function is conserved to human cells

A) Schematic representation of protein structure of *Drosophila* MASK and human ANKHD1 with % identity between sequences.

B) *SOCS3* mRNA expression in HeLa cells transfected with indicated siRNA and following OSM stimulation as indicated. *** $p < 0.001$ (One-way ANOVA with Dunnett's posthoc test).

C) HeLa cell extracts treated with siRNA targeting ANKHD1 have reduced phospho-STAT3 upon OSM stimulation, which total STAT3 levels are unaffected. Blots confirm knockdown of ANKHD1 levels.

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D) A representative blot of HeLa cells treated with control siRNA or siRNA targeting ANKHD1 in (n=3). Silencing of ANKHD1 leads to a loss of both ANKHD1 protein and endogenous GP130 protein. By contrast, levels of LIFR are not changed. β-actin levels are unaffected.

E-F) HeLa cell extracts treated with siRNA targeting ANKHD1 have reduced HA-EpoR (G) and TpoR-HA (H) compared to controls. Blots confirm knockdown of ANKHD1 levels.

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Table 1. Secondary screens confirm 43 robust hits.

After secondary screening with Dome receptor assay, 43 candidates were found to be reproducible hits (see Table S1 and Materials and Methods for further details). Two genes are listed twice, since independent dsRNAs for these were present within the genome plates and taken through to secondary screens. Rab5 was also identified, even though this was used as a positive control. To prove the effectiveness of the STAT reporter assay, we have included results from knockdown of Hop, STAT92E and SOCS36E. Results for MASK are shown in bold. Numbers for receptor dimerisation and STAT reporter assays are shown as Z-scores. Dome protein levels are shown as fold-change relative to controls.

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Table 1

Symbol	Receptor dimerisation assays						STAT reporter assay	Total Dome protein
	Genome		-Upd	+Upd				
Assay:	β-gal	viability	ratio	ratio	ratio	ratio		
Receptors:	D:D		D:D	D:D	D:L	L:L		
CG40121	-6.0	1.6	-8.5	-5.4	-6.5	2.3	-2.3	1
Tor	-5.9	0.3	-2.2	-2.9	-2.6	-3.0	0.7	0.6
MASK	**-2.8**	**4.5**	**-5.5**	**-3.5**	**-3.3**	**-5.2**	**-4.3**	**0.75**
CG7277	-4.4	1.2	-4.1	-2.8	-3.2	0.4	-0.2	0.7
Mi-2	-3.0	2.6	-3.7	-0.8	-2.1	-3.5	2.0	0.3
CG31689	-4.5	0.2	-8.4	-4.1	-8.3	-11.5	0.1	1
Eaf	-3.1	1.3	-3.0	-1.8	-3.4	-5.5	-0.8	0.7
Sec61beta	-2.9	1.4	-2.6	-2.0	-2.6	-4.4	0.4	1
CheB38c	-4.0	-0.4	-3.3	-1.6	-3.5	-4.9	0.4	0.9
Clamp	-4.2	-0.8	-2.9	-2.1	-3.0	-3.7	1.7	0.8
upSET	-2.6	1.6	-2.6	-0.4	-1.8	-2.7	1.3	0.9
CkIalpha	-3.4	0.3	-2.1	-1.7	-2.4	-4.0	5.9	0.9
angel	-3.5	0.2	-3.5	-2.2	-2.9	-2.3	-0.4	1.1
CG6106	-3.4	0.1	-5.3	-3.1	-5.3	-6.2	0.9	1
Hcf	-3.4	-0.1	-3.6	-2.9	-1.7	-1.8	0.1	0.6
Mtor	-3.0	0.3	-2.5	-0.9	-2.8	-4.3	1.0	0.7
GalNAc-T2	-3.5	-0.6	-3.1	-1.5	-3.9	-4.1	1.1	0.6
Jra	-2.5	0.7	-3.9	-0.9	-5.3	-8.8	-0.6	0.6
lig3	-2.9	0.1	-2.3	-2.4	-2.8	-3.5	-0.2	1.1
CG14455	-2.4	0.6	-2.6	-1.1	-2.2	-3.1	0.6	1
tup	-3.0	-0.3	-2.1	-2.5	-2.2	-2.4	1.1	0.7
Fur1	-3.2	-0.6	-2.5	-0.9	-1.4	-1.0	-1.9	0.4
Amt	-3.0	-0.8	-4.3	-3.4	-3.5	-1.1	-1.2	0.5
trr	-3.2	-1.9	-4.7	-2.5	-3.7	-5.1	1.2	0.8
CG34114	-2.8	-1.2	-3.3	-1.4	-3.1	-5.7	0.3	0.5
CG11399	-6.1	-7.0	-6.2	-3.5	-5.6	-8.2	3.3	0.3
CG11399	-1.2	-0.4	-2.0	-0.7	-2.2	-2.8	0.8	1
eff	3.3	3.3	2.6	3.2	2.1	1.7	3.8	1.3
EbpIII	3.1	2.1	4.7	6.5	5.6	9.3	0.7	1.8
SCAR	3.5	2.3	3.1	5.6	3.6	6.0	1.4	2.5
Act87E	2.7	0.4	4.5	3.8	5.3	12.8	-0.4	3.2
CG16772	3.0	0.3	7.9	6.3	8.5	17.5	-0.2	3.9

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eff	3.6	1.0	1.4	3.4	1.6	0.3	2.1	2.1
alphaTub84B	4.0	0.5	4.1	3.3	4.5	11.0	0.5	3
Dp	4.0	0.8	5.8	2.8	4.4	13.1	-1.2	5.3
Sin	3.5	-0.7	4.0	5.8	4.5	7.2	0.7	1.1
Clk	5.0	1.3	2.1	3.3	2.2	2.1	-0.3	3.4
ball	3.6	-1.2	4.0	3.6	4.9	11.2	0.1	2.6
shi	4.7	-0.1	4.0	4.4	4.0	4.8	1.9	1.5
cpa	4.9	0.2	4.0	2.9	3.6	8.4	0.1	2.8
Act42A	6.5	0.8	10.0	7.0	9.6	18.9	-0.5	4.1
PGRP-SC2	6.5	0.1	8.7	5.4	7.8	16.5	0.1	9.9
Rab5	6.7	0.1	5.5	5.6	5.2	7.5	2.0	3.4
Act5C	7.6	0.8	8.4	7.2	10.0	18.3	0.3	3.9
CG4511	6.9	-0.8	6.5	4.3	5.1	10.5	-0.1	10
tsr	7.5	-0.3	10.2	7.1	9.6	16.9	0.3	9.5
hop	0.5	0.2	0.0	-0.7	0.0	-0.1	-7.4	1
Stat92E	0.1	-0.6	0.7	-0.6	0.2	0.2	-7.0	0.8
Socs36E	1.2	-0.2	-0.5	-1.2	-1.0	-1.0	4.7	0.9

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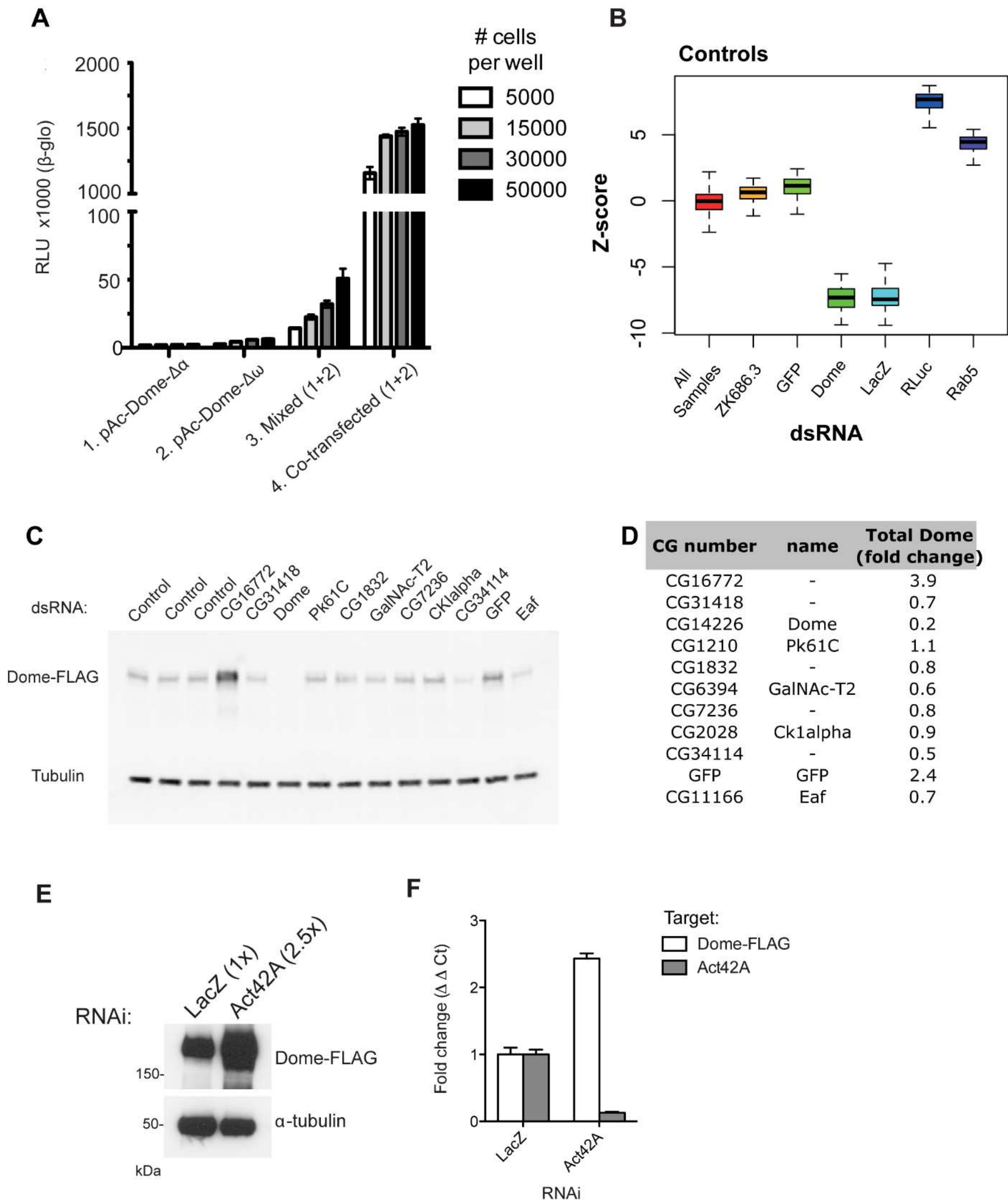
Supplemental Figure 1



CG number	name	Total Dome (fold change)
CG16772	-	3.9
CG31418	-	0.7
CG14226	Dome	0.2
CG1210	Pk61C	1.1
CG1832	-	0.8
CG6394	GalNAc-T2	0.6
CG7236	-	0.8
CG2028	Ck1alpha	0.9
CG34114	-	0.5
GFP	GFP	2.4
CG11166	Eaf	0.7

Figure S1. A) A significant increase in β-gal activity is observed in cells co-transfected with both plasmids (4), compared to cells transfected with individual plasmids and mixed together (3), demonstrating the specificity of the assay. **B**) Box-and-whisker plots showing Z-scores of negative control RNAi (*C.elegans* gene *ZK686.3* or *GFP*) show little variation from the median, whereas technical positive RNAi controls, targeting the transfected plasmids (*Dome, LacZ, RLuc*) show significant Z-scores. Further positive control (Rab5), targeting the endocytic machinery and causing an increase in Dome stability, shows a significant increase in enzyme activity. **C-D**) Example western blot (C) and quantification (D) from secondary RNAi screen analysis, measuring Dome protein levels. **E**) Dome-FLAG protein levels increase in Kc_{167} cells upon knockdown of *Act42A*, resulting in a 2.5-fold increase compared to a *LacZ* control. **F**) qPCR of the Dome-FLAG construct also shows an approximately 2.5-fold increase in expression upon knockdown of *Act42A*. Efficiency of RNAi is confirmed by qPCR of *Act42A*.

Supplemental Figure 2

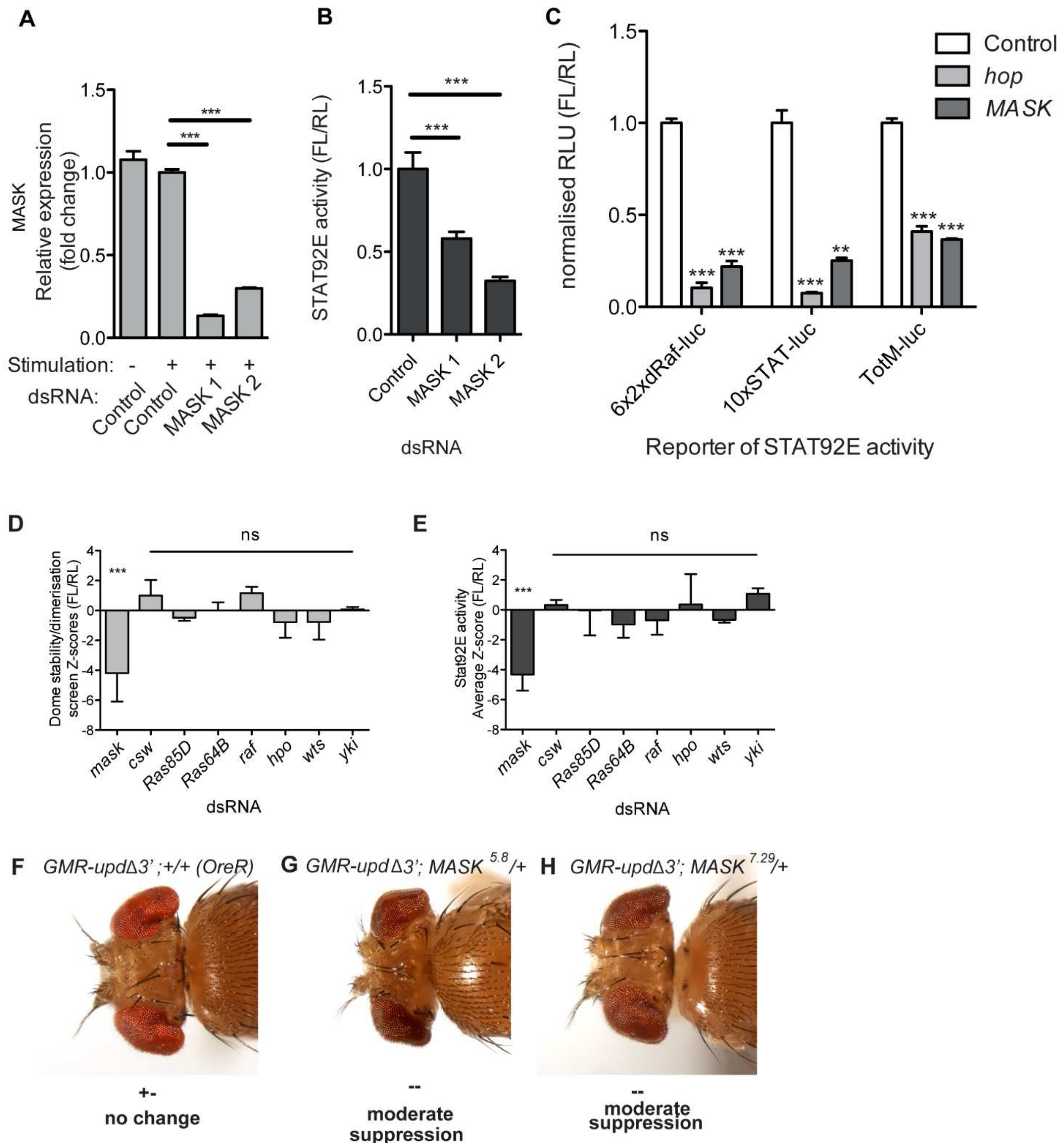


Figure S2. A) mRNA expression levels of *MASK* in Kc_{167} cells assayed by qPCR after indicated RNAi treatment (MASK1= BKN20625; MASK2 = HFA16018) relative to housekeeping gene RpL32. Knockdown of *MASK* levels are confirmed. *** $p < 0.01$. **B**) *6x2xdRafluc* STAT92E reporter assay is reduced after indicated RNAi treatment. **C**) Three different STAT92E-dependent luciferase reporters were used to measure JAK/STAT activity after stimulation with Upd. Significant changes were observed after indicated RNAi treatment for all STAT92E-dependent reporters. **D**) Z-scores derived from the Dome dimerisation genome-scale RNAi screen comparing the effect of MASK knockdown (column 1) to the Ras/Raf pathway components *csw, Ras85D, Ras64B, raf* and for the Hipo pathway genes *hpo, wts* and *yki*. None of the interactions were significant (ns). **E**) Z-scores derived from a previous genome-scale RNAi screen for modulators of the *6x2xDrafLuc* STAT92E activity reporter {Fisher et al., 2012, BMC Genomics, 13, 506}. The effect of MASK (column 1) is compared to the Ras/Raf pathway genes *csw, Ras85D, Ras64B, raf* and to the Hippo pathway genes *hpo, wts, yki*. None of the interactions were significant (ns). **F-H**) Dorsal view of eye overgrowth phenotypes caused by ectopic Upd ligand expression driven by *GMR-UpdΔ3'.* Panels show an alternative control (OreR), which was scored as normal, and two further MASK alleles (*MASK*$^{5.8}$ and *MASK*$^{7.29}$), which were scored as having moderate suppression.

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Supplemental Figure 3

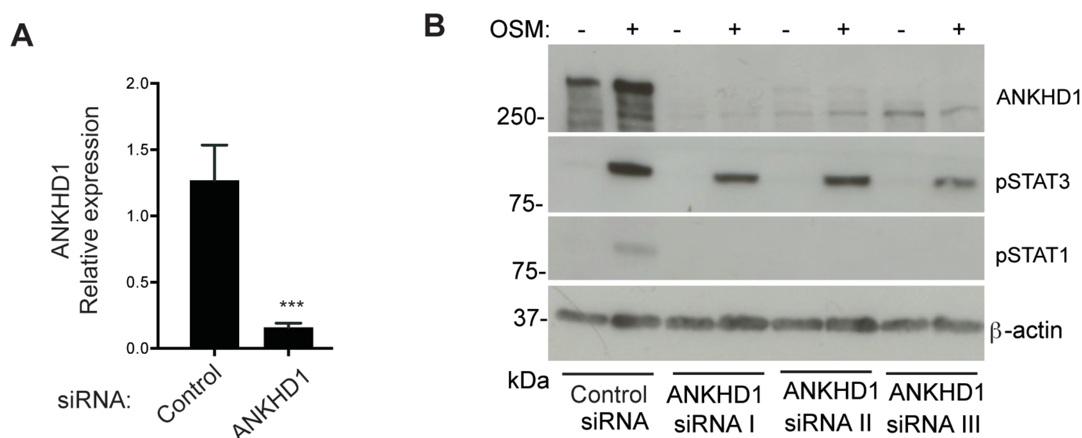

Figure S3
A) mRNA expression of ANKHD1 indicated from HeLa cells, after siRNA treatment of ANKHD1or non-targeting control. Measurements were taken relative to β-actin.
B) phospho-STAT1 (pSTAT1) and phospho-STAT3 (pSTAT3) protein are increased by ligand stimulation (OSM) in HeLa cells treated with control siRNA. Induction of phosphorylated STATs was suppressed when treating cells with non-overlapping siRNA reagents targeting ANKHD1. β-actin was used as a loading control.

Table S1. Full table of hits selected from genome-wide RNAi screen.

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