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Report

Ken & Barbie Selectively Regulates the Expression of a Subset of JAK/STAT Pathway Target Genes

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Summary

A limited number of evolutionarily conserved signal transduction pathways are repeatedly reused during development to regulate a wide range of processes. Here we describe a new negative regulator of JAK/STAT signaling and identify a potential mechanism by which the pleiotropy of responses resulting from pathway activation is generated in vivo. As part of a genetic interaction screen, we have identified Ken & Barbie (Ken) [1], which is an ortholog of the mammalian proto-oncogene BCL6 [2], as a negative regulator of the JAK/STAT pathway. Ken genetically interacts with the pathway in vivo and recognizes a DNA consensus sequence overlapping that of STAT92E in vitro. Tissue culture-based assays demonstrate the existence of Ken-sensitive and Ken-insensitive STAT92E binding sites, while ectopically expressed Ken is sufficient to downregulate a subset of JAK/STAT pathway target genes in vivo. Finally, we show that endogenous Ken specifically represses JAK/STAT-dependent expression of *ventral veins lacking (vvl)* in the posterior spiracles. Ken therefore represents a novel regulator of JAK/STAT signaling whose dynamic spatial and temporal expression is capable of selectively modulating the transcriptional repertoire elicited by activated STAT92E in vivo.

Results and Discussion

Ken Genetically Interacts with JAK/STAT Signaling

Analysis of phenotypes associated with mutations in *Drosophila* JAK/STAT pathway components have identified a wide variety of requirements for the pathway during embryonic development and in adults (reviewed in [3, 4]). What is less clear is how the repeated stimulation of a single pathway is able to generate this pleiotropy of developmental functions. In order to identify modulators of JAK/STAT signaling that may be involved in this process, we undertook a genetic screen for modifiers of the dominant phenotype caused by the ectopic expression of the pathway ligand Unpaired (Upd) in the developing eye imaginal disc. Such misexpression by *GMR-updΔ3′* results in overgrowth of the adult eye, a phenotype sensitive to the strength of pathway signaling activity [5]. With this assay, one genomic region, defined by *Df(2R)Chi^{g320}*, was found to enhance the *GMR-updΔ3′*-induced eye overgrowth phenotype. Of the genes deleted by *Df(2R)Chi^{g320}*, only mutations in *ken* showed consistent and reproducible enhancement of the phenotype (Figures 1A and 1B and not shown). In addition, other dominant phenotypes induced by transgene expression from the GMR promoter are not modulated by *ken* mutations, indicating that Ken is unlikely to interact with the misexpression construct used (not shown).

The enhancement of the *GMR-updΔ3′* phenotype after removal of one copy of *ken* implies that Ken normally functions antagonistically to JAK/STAT signaling. We therefore tested phenotypes associated with mutations in other pathway components to establish the reliability of this initial observation. Consistent with this, genetic interaction assays between *ken* mutations and the hypomorphic loss-of-function allele *stat92E^{HJ}* [6] show a reduction in the frequency of wing vein defects normally associated with this *stat92E* allele (Figures S1A and S1B in the Supplemental Data available with this article online). Moreover, the degree of suppression is consistent with the strength of *ken* alleles tested (Supplemental Data and Table S1). Similarly, the frequency of "strong" posterior spiracle phenotypes caused by the *dome^{367}* allele of the pathway receptor is also reduced when crossed to *ken* alleles or the *Df(2R)Chi^{g320}* deficiency, with a concomitant increase in "weak" phenotypes (Table S2 and Figures S1C–S1E).

Thus, multiple independent *ken* alleles all modify diverse phenotypes caused by both gain- and loss-of-function mutations in multiple JAK/STAT pathway components. Each of these components acts at different levels of the signaling cascade and show interactions indicating that Ken consistently acts as an antagonist of the pathway.

The Ken Locus

The *ken* locus contains three exons encoding a 601 aa protein (Figure 1C) [1, 7, 8]. Ken possesses an N-terminal BTB/POZ domain between aa 17 and 131 and three C-terminal C2H2 zinc finger motifs from aa 502 to 590 (Figure 1D). Strikingly, a number of Zn finger-containing proteins that also contain BTB/POZ domains have also been shown to function as transcriptional repressors—often via the recruitment of corepressors such as SMRT, mSIN3A, N-CoR, and HDAC-1 [9, 10].

Searches for proteins similar to Ken identified homologs in *Drosophila pseudoobscura* and the mosquito *Anopheles gambiae*. In vertebrates, human B-Cell Lymphoma 6 (BCL6) [11] was the closest full-length homolog. *Drosophila* Ken and human BCL6 share the same

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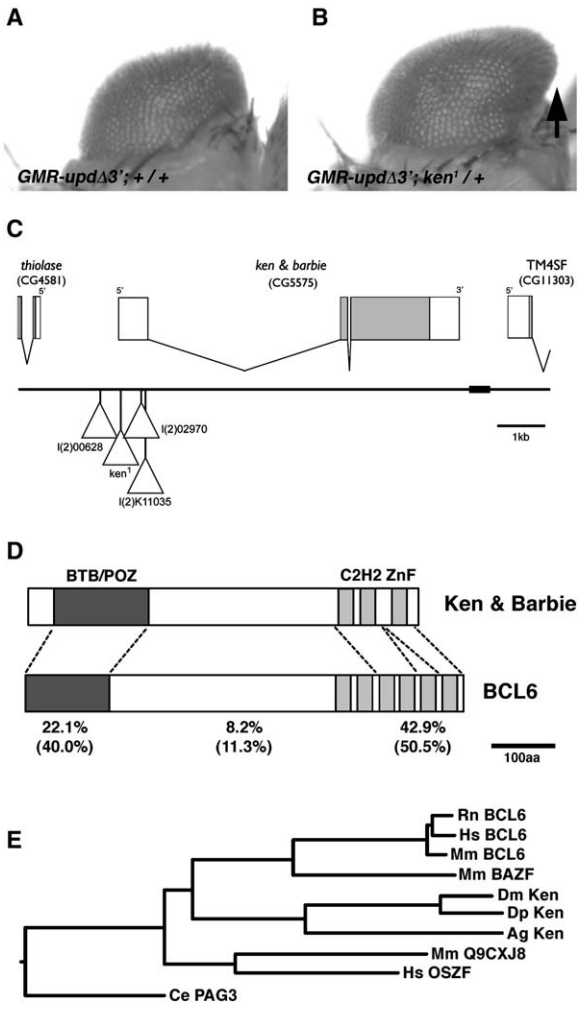

Figure 1. Genetic Interactions between *ken* and the JAK/STAT Pathway

(A and B) Dorsal views of adult *GMR-upd Δ3'* flies either wild-type (A) or heterozygous for the *ken*^{1} allele (B). Note the additional overgrowth in the eye caused by the removal of one copy of *ken* (arrow in B).

(C) The *ken* genomic region is flanked by the *thiolase* and *TM4SF* loci. The position of P element insertions that give rise to the alleles used are shown (not to scale). Coding regions are shown in gray and include three amino acids encoded by exon 1.

(D) The predicted Ken protein contains an N-terminal BTB/POZ domain and three C-terminal zinc fingers. The degree of identity (similarity) to human BCL6 is shown.

(E) A phylogenetic tree showing the relationship of human (Hs), mouse (Mm), and rat (Rn) BCL6 genes to BAZF and the related invertebrate Ken-like proteins from *Drosophila melanogaster* (Dm), *D. pseudobscura* (Dp), and mosquito (Ag). More distantly related genes from human, mouse, and *C. elegans* (Ce) are also shown.

domain structure and show 20.3% overall identity (Figure 1D) [12]. Proteins listed as potential vertebrate homologs of Ken in Flybase (http://flybase.bio.indiana.edu) are more distantly related (Figure 1E).

Expression of *ken* was also examined during development, where it is detected in a dynamic pattern from newly laid eggs, throughout embryogenesis (Figures 2A–2E), and in imaginal discs (Figures 2H–2J). As such, endogenous Ken is present in all tissues and stages in which genetic interactions were observed.

Ken Binds to a DNA Sequence that Overlaps that of STAT92E

Given the presence of potentially DNA binding Zn finger domains and the nuclear localization of GFPKen (Figures 2K–2N), we set out to determine the DNA binding properties of Ken by using an in vitro selection technique termed SELEX (systematic evolution of ligands by exponential enrichment) [13]. With a GST-tagged Ken Zn finger domain and a randomized oligonucleotide library, ten successive rounds of selection were undertaken. Sequencing of the resulting oligonucleotide pool and alignment of 43 independent clones showed that all recovered plasmids were unique and each contained one, or occasionally two, copies of the motif GNGAAAK (K = G/T; Table S3).

To confirm the SELEX results, we expressed GFPKen in tissue culture cells and used these for electromobility shift assays (EMSA). A radioactively labeled probe containing the wild-type (wt) consensus binding site GAG AAAG gave a specific band, which can be supershifted by an anti-GFP antibody (Figure 3A) and therefore represents a GFPKen/DNA complex. In order to identify positions essential for binding, we used a competition assay in which unlabeled oligonucleotides containing single substitutions in each position from 1 to 7 (Table S3) were added to binding reactions. 10-fold excess of unlabeled wild-type consensus oligonucleotide greatly diminished the intensity of the GFPKen band, while 50- and 100-fold excess totally blocked the original signal (Figure 3B). By contrast, competition with unlabeled *m3* oligonucleotides containing a G to A substitution at position 3 failed to significantly reduce the intensity of the band even at 100-fold excess (Figure 3B). With this approach, the positions 1 and 7 are found dispensable for DNA binding, whereas the central GAAA core is absolutely required (Figure 3B). Similar results were obtained with the converse experiment with labeled mutant probes, although in this case the wt probe produces a stronger signal than the *m1* and *m7* mutant oligonucleotides (not shown). Taken together, these experiments not only define the core sequence for Ken binding, but also demonstrate the specificity of Ken as a site-specific DNA binding molecule.

Interestingly, the core consensus bound by Ken is very similar to that identified for human BCL6, with the Zn fingers of the latter binding to a DNA sequence containing a core GAAAG motif [12, 14].

Ken Selectively Regulates JAK/STAT Reporters in Cell Culture

One initial observation made is that the core GAAA essential for Ken binding overlaps the sequence recognized by STAT92E (Figure 3C) [15]. Consistent with this overlap, a 100-fold excess of unlabeled oligonucleotide containing the STAT92E consensus is sufficient to fully compete for Ken in EMSA assays (Figure 3B). Given this finding, we hypothesize that the negative regulation of JAK/STAT signaling by Ken observed in genetic interaction assays may occur via a mechanism of competitive DNA binding site occupation. Due to the incomplete overlap between the STAT92E and Ken core sequences (Figure 3C), this hypothesis also implies the existence

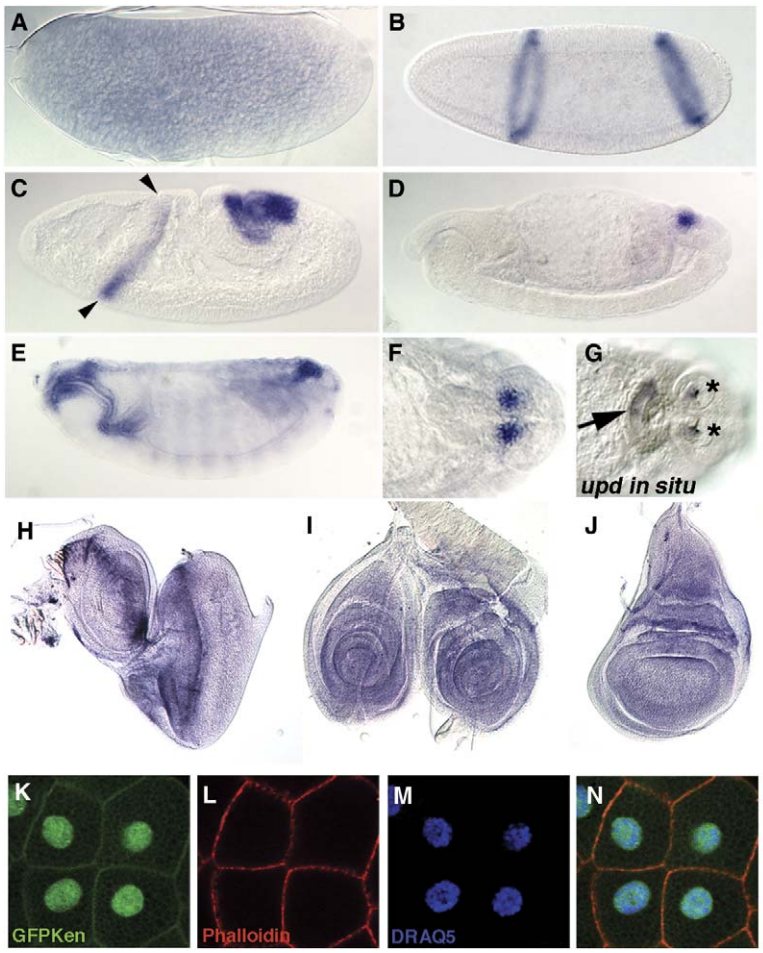

Figure 2. *ken* Expression during Development

(A–F) Embryonic *ken* expression. In the stage 3/4 blastoderm (A), low levels of maternally supplied *ken* are detected that resolve to two narrow stripes of expression (at 64% and 16% of egg length) by stage 5 (B). At the onset of gastrulation, the anterior stripe immediately posterior to the cephalic furrow (arrowheads), weakens, while the posterior domain coalesces (C). *ken* is then expressed in the presumptive hindgut/posterior spiracles and maintains its relative position within the extending germband. By stage 13, only the posterior spiracle primordia express *ken* (D). At stage 15 and later, *ken* is visible in the foregut and posterior spiracles, within the posterior region of the dorsal tracheal trunk, and in segmentally repeated stripes within the epidermis (E).

(F and G) Dorsal views of stage 13 embryos showing expression of *ken* (F) and *upd* (G). Both *ken* and *upd* are expressed in the presumptive posterior spiracles, though the region of *upd* expression (asterisks) is smaller and occupies only the center of the primordia. Arrow marks the expression of *upd* in the hindgut.

(H–J) *ken* expression in eye-antennal (H), leg (I), and wing (J) imaginal discs from late third instar larvae.

(K–N) Intracellular localization of GFPKen expressed by *sgs3-GAL4* in the third instar salivary gland. GFPKen (green) is primarily located in the nuclei identified by the DNA stain DRAQ5 (blue) and is enriched at the cell membrane. Cell morphology is visualized by TRITC-phalloidin (red), which stains subcortical F-actin. All three channels are shown separately (K–M) and merged (N).

of STAT92E DNA binding sites to which both STAT92E and Ken could bind ($STAT^+/Ken^+$) as well as sites with which Ken cannot associate ($STAT^+/Ken^-$) (Figure 3D).

To test this hypothesis, we set up a cell culture-based assay by using a luciferase-expressing reporter containing four STAT92E binding sites (Figure 3D) originally identified in the promoter of the *Draf* locus [16]. In addition to this $STAT^+/Ken^+$ wild-type reporter, we also generated $STAT^+/Ken^-$ and $STAT^-/Ken^-$ variants identical but for the binding sequences shown in Figure 3D. When transfected into the hemocyte-like Kc_{167} *Drosophila* cell line, both $STAT^+/Ken^+$ and $STAT^+/Ken^-$ reporters showed strong stimulation upon coexpression with the pathway ligand Upd (Figure 3E), an assay previously shown to require an intact JAK/STAT cascade [17]. When cotransfected with KenGFP, the activity of the $STAT^+/Ken^+$ reporter was reduced (Figure 3E), an effect reproduced in three independent experiments with both KenGFP and Ken (not shown). While the reduction in reporter activity for the $STAT^+/Ken^+$ assay shown is statistically significant ($p = 0.007$), the $STAT^+/Ken^-$ reporter was unaffected by the coexpression of Ken (Figure 3E). Reporters containing binding sites mutated to prevent binding of both STAT92E and Ken ($STAT^-/Ken^-$) showed no activation after pathway stimulation and did not respond to Ken (Figure 3E).

These results indicate that Ken functions as a transcriptional repressor in this cell-culture system and shows that this effect is specific to the DNA sequence determined by SELEX and EMSA. This result is also consistent with a recent whole-genome RNAi-based screen, which used a reporter containing $STAT^+/Ken^+$ binding sites and includes Ken among the list of JAK/STAT regulators identified [18]. In addition, recent reports have also demonstrated BCL6 binding to STAT6 sites in vitro and have shown that BCL6 can act as a repressor of STAT6-dependent target gene expression in cell culture [19, 20]. Although this repression is mediated by the binding to corepressors to the BTB/POZ domain of BCL6 [9], no link between BCL6 and STAT activity has been demonstrated in vivo.

Finally, it should also be noted that both the $STAT^+/Ken^+$ and $STAT^+/Ken^-$ reporters contain additional GAAA sequences that are not part of the characterized STAT92E binding sequences. However, despite the presence of these potential Ken binding sites within 15 bp of the STAT92E site, Ken expression did not affect the $STAT^+/Ken^-$ reporter (Figure 3E), suggesting that Ken may require STAT92E to influence gene expression. Although we have not been able to demonstrate a direct association between Ken and STAT92E (not shown), we cannot exclude this possibility, and further analysis remains to be undertaken.

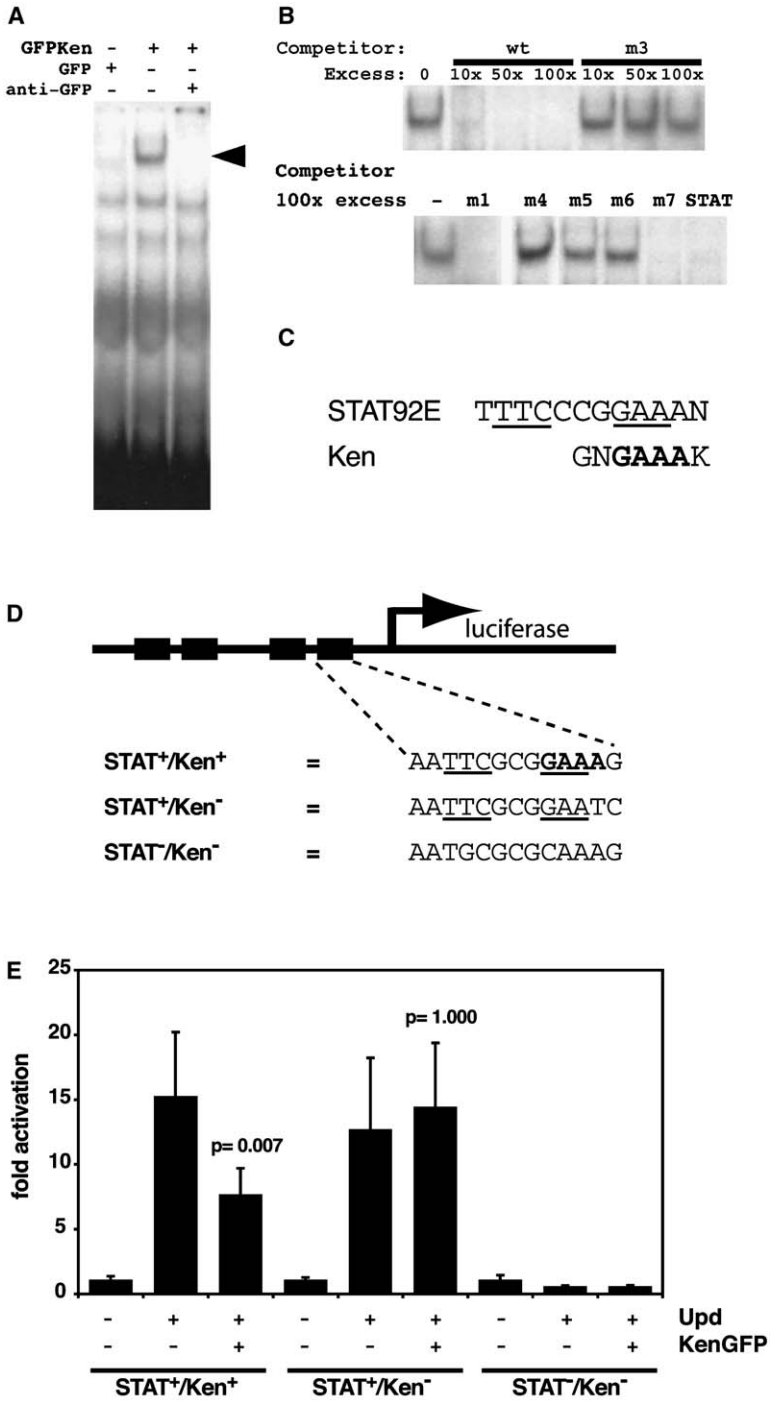

Figure 3. Ken DNA Binding and Reporter Assays

(A) GFPKen and GFP were derived from S2R+ cell lysates. GFPKen specifically binds to the radioactively labeled probe containing the GAGAAAG consensus sequence. The resulting band (arrowhead) can be supershifted by anti-GFP antibody.

(B) Unlabeled binding site competition assays. Wild-type oligonucleotides include the GAGAAAG consensus. *m1*, *m3*, and *m7* contain G to A substitutions in positions 1, 3, and 7, respectively. *m4*, *m5*, and *m6* have A to T substitutions in the corresponding positions. Loss of the specific band indicates that the indicated excess of unlabeled mutant oligonucleotides are capable of binding to Ken.

(C) A schematic representation of STAT92E (underlined) and Ken (bold) consensuses binding sites.

(D) Schematic of the *2x2DrafLuc* reporter plasmids showing the four identical STAT92E binding domains (larger blocks) located upstream of the firefly luciferase ORF. The sequences of the STAT92E binding regions are shown, with the minimal STAT92E binding sequence underlined and the minimal Ken binding sequence shown in bold. Not shown to scale.

(E) Effects of cotransfection of Upd- and KenGFP-expressing plasmids on luciferase reporter activity. *Drosophila* Kc_{167} cells were cotrasfected with the indicated reporters (for sequences, see [D]). Values represent the ratio of firefly luciferase to a constitutively expressed *Renilla* luciferase transfection control and are shown as multiples of the unstimulated cells. One representative experiment undertaken in sextuplicate is shown. Error bars show standard deviations, and p values are calculated by a Student's t test comparing Upd-expressing with Upd- and Ken-expressing cells.

Ken Is Sufficient to Downregulate a Subset of JAK/STAT Pathway Target Genes In Vivo

Having established that Ken functions at the level of DNA binding in cell culture, we asked whether Ken also acts as a transcriptional repressor of JAK/STAT pathway target genes in vivo. For this, we examined the effect of ectopically expressed Ken on the expression of putative JAK/STAT pathway target genes and, given the high levels of maternally loaded STAT92E present at blastoderm stage, we focused on targets expressed later in embryogenesis. These include the hindgut-specific expression of *vvl* [21], the expression of *trachealess* (*trh*) and *knirps* (*kni*) in the tracheal placodes [22], and the dynamic expression of *socs36E* throughout the embryo [23].

First, we addressed the effect of Ken on *trh*, expression of which precedes the formation of the tracheal pits in the embryonic segments T2 to A8 (Figures 4A and 4D) [22]. Levels of *trh* are greatly reduced in embryos uniformly misexpressing Ken driven by the *daughterless-GAL4* (*da-GAL4*) line (Figure 4B). Many tracheal placodes express little or no *trh*, and tracheal pits fail to form even in the presence of residual *trh* (Figure 4E). Similar effects are seen in upd^{OS1A} mutant embryos

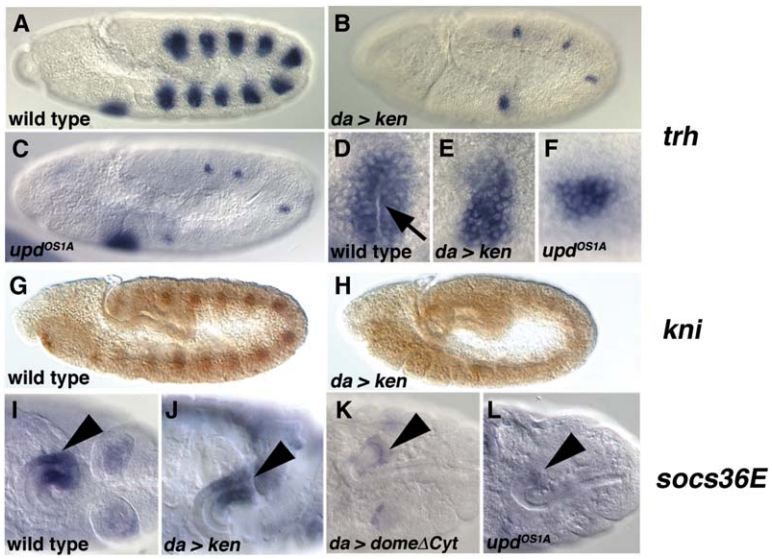

Figure 4. JAK/STAT Target Genes and Ken

(A–F) *trh* expression in stage 10–11 embryos of the indicated genotypes.

(A) Wild-type embryos show expression in ten tracheal placodes and the presumptive salivary gland.

(B) In *da-GAL4/UAS-ken* (*da > ken*), only weak staining is seen in a few tracheal placodes.

(C) Low levels of *trh* expression are also observed in embryos mutant for the strong upd^{OS1A} allele.

(D–F) High-magnification views of the first tracheal placode. Tracheal pits formed in wild-type embryos (arrows in [D]) are missing in both *da > ken* (E) and upd^{OS1A} mutants (F).

(G and H) Stage 10/11 embryos stained with anti-Knirps antibody. Staining in the tracheal pits typical for wild-type embryos (G) is not detectable in embryos expressing *da > ken* (H).

(I–L) Expression of *socs36E* in the hindgut of stage 13 embryos (arrowheads). Expression in the wild-type embryo (I) is similar to that in *da > ken* embryos (J). By contrast, significantly reduced levels of *socs36E* are detected in the *da > domeΔCyt* (K) and upd^{OS1A} mutant embryos (L).

lacking all pathway activity (Figures 4C and 4F). Likewise, downregulation of Kni expression is also observed in embryos misexpressing *ken* (Figures 4G and 4H). These results show that both endogenous *trh* and *kni* are downregulated by ectopically expressed Ken.

We then tested whether Ken can modulate the expression of *socs36E*, a *Drosophila* homolog of mouse SOCS-5. *socs36E* expression closely mirrors that of *upd*, showing JAK/STAT pathway-dependent upregulation in segmentally repeated stripes, tracheal pits, and the hindgut [23]. By contrast to *trh* and *kni*, ectopically expressed Ken does not affect any aspect of *socs36E* transcription (Figures 4I and 4J and not shown). However, controls expressing a dominant-negative form of the pathway receptor DomeΔCyt [22], using the same Gal4 driver line, show a strong downregulation of *socs36E* (Figure 4K), an effect reproduced by the complete removal of all JAK/STAT pathway activity by the upd^{OS1A} allele (Figure 4L).

Taken together, these results illustrate that ectopic expression of Ken during *Drosophila* development is sufficient to downregulate the expression of only a subset of putative JAK/STAT pathway target genes.

Ken Regulates STAT92E-Induced *vvl*

As part of our analysis, we tested for modulation of *vvl* by Ken. In wild-type embryos, *vvl* is expressed in the developing trachea and lateral ectoderm (in a JAK/STAT-independent manner) and in the hindgut of stage 12–14 embryos (Figure 5A), where it requires JAK/STAT signaling [21]. In upd^{OS1A} mutants, no *vvl* expression in the hindgut can be detected ([21] and not shown), indicating that this locus is a target of pathway activation. When Ken is uniformly misexpressed throughout the embryo, *vvl* expression is no longer detectable in the hindgut (Figure 5C). Thus *vvl*, like *trh* and *kni*, can be a target of Ken-mediated repression.

Having established that ectopic Ken is sufficient to downregulate *vvl* in the hindgut, we set out to determine whether endogenous Ken performs a similar role. One overlap between *ken* expression and regions known to require JAK/STAT signaling are the developing posterior spiracles, structures in which both the pathway ligand *upd* and *ken* are simultaneously expressed (Figures 2F and 2G). However, *vvl* is never detected in the posterior spiracle primordia in wild-type embryos (Figure 5B), despite JAK/STAT pathway activity induced by *upd* expression in these tissues [21]. Intriguingly, in a heteroallelic combination of the strongest ken^{k11035} allele and $Df(2R)Chi^{g320}$, *vvl* transcript was detected not only in its normal expression domain within the hindgut (Figure 5E) but also in the posterior spiracles (Figure 5F). This ectopic expression is initially detected from late stage 13 and rapidly strengthens during stage 14–15. When $ken^{k11035}/Df(2R)Chi^{g320}$ embryos simultaneously mutant for the amorphic upd^{OS1A} allele were analyzed, upregulation of *vvl* in the presumptive posterior spiracles was never observed at the stage by which ectopic *vvl* expression was first detected in the *ken* mutant embryos (not shown). At later stages, JAK/STAT pathway activity is required for posterior spiracle morphogenesis [21, 24], posterior spiracles do not form, and upregulated *vvl* is not present (Figure 5H).

These results demonstrate that Ken is not only sufficient to downregulate the JAK/STAT pathway-dependent expression of *vvl* in the hindgut, but its endogenous expression is also necessary for *vvl* repression in the posterior spiracles. In *ken* mutants, ectopic *vvl* expression in the posterior spiracles results from a derepression of endogenous STAT92E activity.

Ken as a Selective Transcriptional Regulator

The overlap between the consensus sequences bound by STAT92E and Ken, together with the analysis of reporters containing $STAT^{+}/Ken^{+}$ and $STAT^{+}/Ken^{-}$

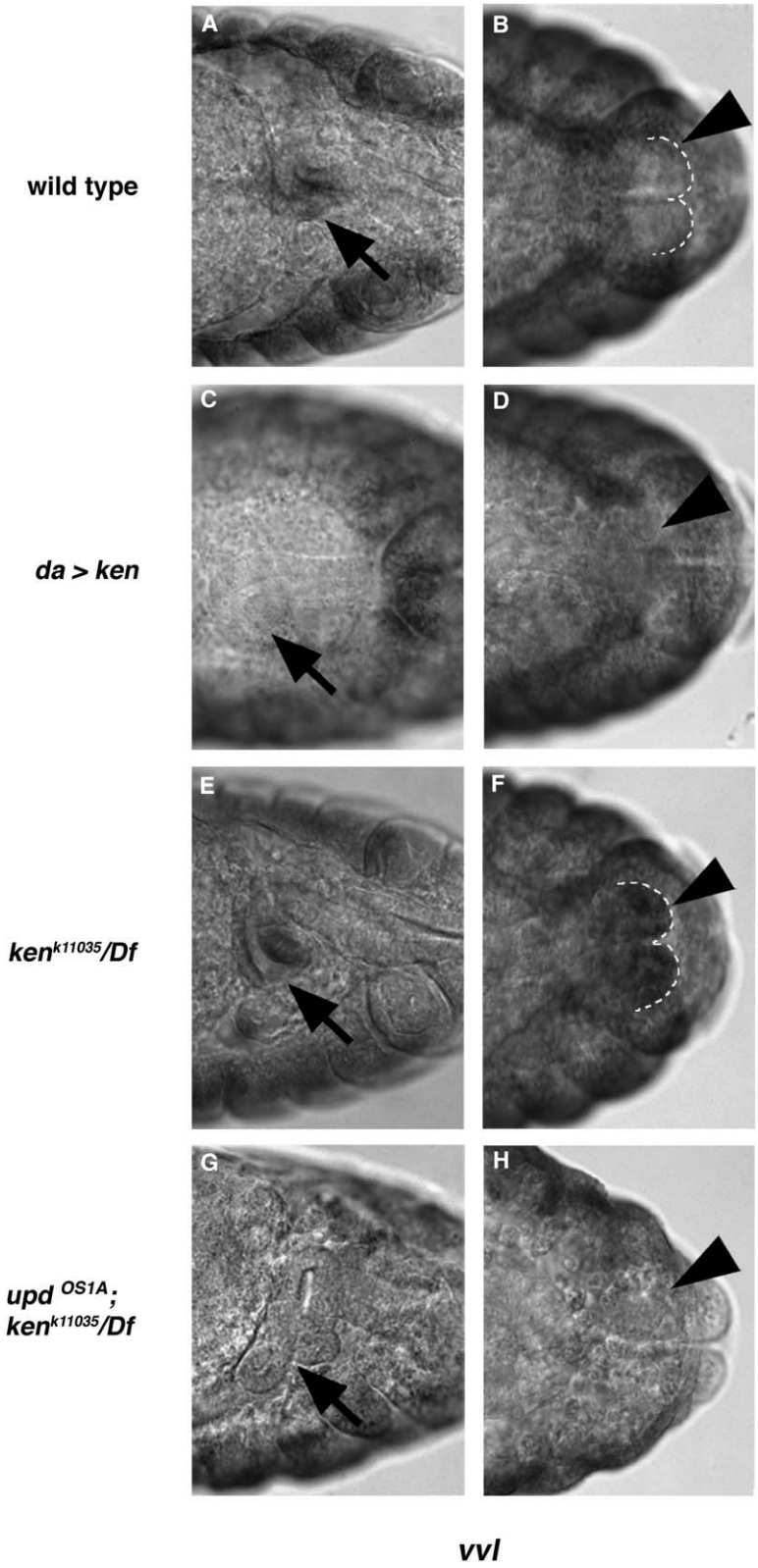

Figure 5. Ken Is Necessary and Sufficient to Regulate Expression of *vvl*

vvl expression in the hindgut (arrows in [A], [C], [E], and [G]) of stage 13 embryos and in the presumptive posterior spiracles (arrowheads in [B], [D], [F], and [H]) of stage 14 embryos. Genotypes are indicated on the left where *da > ken* is *da-GAL4/UAS-ken* and *Df* is $Df(2R)Chi^{g230}$. Note that in wild-type embryos, *vvl* is expressed in the hindgut (A), but not in the posterior spiracles (outlined in [B]). In *da > ken* embryos, *vvl* expression is downregulated in the hindgut (C) and remains repressed in the poorly differentiated spiracle Anlagen (D). In $ken^{k11035}/Df(2R)Chi^{g230}$ transhererozygotes, *vvl* is detected in the hindgut (E) as in wild-type (A) and is upregulated in the posterior spiracles (outlined in [F]). In upd^{OS1A}; $ken^{k11035}/Df(2R)Chi^{g230}$ double mutants, *vvl* cannot be detected in the hindgut (G). Similarly, no expression is detected in the region normally occupied by the presumptive posterior spiracles that do not form properly in this genotype (H).

binding sites, indicate that Ken is likely to selectively regulate only a subset of JAK/STAT target genes. In this model, some target genes are regulated by binding sites compatible with both STAT92E and Ken, while others contain sequences to which only STAT92E can associate. While the DNA binding site is critical in cell-culture systems, similar proof is more difficult to establish in vivo. In particular, only a limited number of JAK/STAT pathway target genes have been rigorously demonstrated to require STAT92E binding in vivo [15, 16, 25].

Although studied in some detail, the regulatory domains controlling *vvl* expression in the developing hindgut have not been identified [26, 27]. Therefore, although our results predict that such a domain would contain $STAT^{+}/Ken^{+}$ binding sequences, further analysis is required to confirm this hypothesis. By contrast, the regulatory domain of *socs36E* required to drive gene expression in the blastoderm, tracheal pits, and hindgut comprises a 350 bp region containing three $STAT^{+}/Ken^{+}$ and two $STAT^{+}/Ken^{-}$ binding sites (M.P.Z., unpublished data). Although not conclusive, the presence of STAT92E-exclusive sites in this region may explain the inability of Ken to downregulate *socs36E* in vivo (Figures 4I and 4J).

Our findings also draw a parallel between *Drosophila* Ken and BCL6. The data presented demonstrate that both proteins show similar abilities to bind DNA and to mediate transcriptional repression with some evidence also linking BCL6 to JAK/STAT signaling as described here. Taken together, these similarities suggest that Ken and BCL6 represent functional orthologs of one another. Given this evolutionary conservation, it is tempting to speculate that the selective regulation of JAK/STAT pathway target genes is also conserved and may represent a general mechanism by which the pathway is modulated to elicit diverse developmental roles in vivo. Although many STAT targets undoubtedly remain to be identified, it will be intriguing to see which may also be coregulated by Ken/BCL6-dependent mechanisms.

Experimental Procedures

Genetics

Fly stocks used were: Ore R, *GMR-updΔ3′* [5], *Df(2R)Chi^{g320}* [28], *ken^{1}* [29], *ken^{02970}* and *ken^{k11035}* [7], *stat92E^{06342}* [30], *stat92E^{HJ}* [6], *sgs3-GAL4* [31], *da-GAL4* [32], *dome^{468}* and *dome^{367}* [22], and *Df(1)os^{1A}* (referred to as *upd^{OS1A}*) [33]. All flies, larvae, and embryos were grown at 25ºC, except for those shown in Figures 1A and 1B, which were grown at 18ºC.

ken alleles were tested for interaction with *GMR-Rho1* and *GMR-yan* [34, 35]. For *stat92E^{HJ}* interactions, both extra and missing wing veins were scored as phenotypic.

DNA Constructs

ken cDNA was derived from EST clones *GH12495* and *LD29702*. For misexpression constructs, full-length *ken* ORF was amplified by PCR and subcloned into *pUAST* [36]. EGFP-Ken fusions were generated by cloning into *pBS-EGFPA* or *pBS-EGFPB* [37] followed by subcloning into *pUAST* and *pAc5.1A* (Invitrogen). To tag with GST, Ken DNA binding domain (aa 475 to 601) was amplified and cloned into *pGEX-2T* (Amersham). All PCR reactions were carried out with *PfuTurbo* (Stratagene) and confirmed by DNA sequencing.

For luciferase assays, the *$STAT^{+}/Ken^{+}$* sites in *2xDrafSTATwt-TATA-luc* [16] were duplicated by reinsertion of a 165 bp blunted *BamH*I-*Xba*I fragment into a *Sma*I site immediately upstream of the original binding sites to give *2x2DrafLuc($STAT^{+}/Ken^{+}$)*. QuikChange (Stratagene) mutagenesis was used to generate $STAT^{+}/Ken^{-}$ and $STAT^{-}/Ken^{-}$ variants resulting in *2x2DrafLuc($STAT^{+}/Ken^{-}$)* and *2x2DrafLuc($STAT^{-}/Ken^{-}$)*.

Histology

Salivary glands and embryos were prepared by standard techniques and stained with rabbit anti-Knirps (1:200) [38], TRITC-phalloidin (Sigma), and DRAQ5 (Biostatus). Whole-mount in situ hybridization was performed as described previously [39] by RNA probes prepared from *pCRII-ken*, *pCRII-vvl*, *pFLC-1-trh*, *pOT2-RE54280* (BDGP), and *pBS-lacZ*. Embryonic cuticles were prepared as described previously [40]. Pictures shown in Figures 2K–2N were captured on a Leica TCS SP2 confocal microscope in sequential scan mode to exclude the possibility of interchannel crosstalk.

SELEX

For SELEX [13], GST-KenZnF was purified from *E. coli* grown in LB medium supplemented with 1 μM $ZnCl_2$. The binding site library contained 20 bp random nucleotides. 80 ng of GST-KenZnF bound to glutathione beads was allowed to associate with 320 ng of library representing a complexity of approximately 10×. Protein/DNA complexes were allowed to form in SELEX binding buffer (20 mM HEPES [pH 7.9], 40 mM KCl, 50 μM $ZnSO_4$, 1.4 mM $MgCl_2$, 0.1 mM EGTA, 5% Glycerol, 1 mg/ml BSA, 1× Complete EDTA-free Protease Inhibitor Cocktail [Roche], 50 μg/ml poly-dI-dC, 0.5 mM DTT, and 1 mM PMSF) for 20 min. One-quarter of the bound and recovered DNA was amplified by 20 cycles of PCR and purified via electrophoresis through 4% MetaPhor agarose (Cambrex). This DNA was used as the starting point for the next selection cycle. After 10 rounds of selection, amplified DNA was subcloned into *pCRII-TOPO* (Invitrogen), and 43 independent clones were sequenced. Sequences were aligned by Gibbs Sampler and manual techniques.

Cell Culture and Transfection

S2R+ and Kc_{167} cells were maintained in Schneider's *Drosophila* medium (GIBCO) + 10% fetal calf serum at 25ºC. For EMSA, S2R+ cells in 10 cm dishes were cotransfected with 400 ng *pUC18-Act-GAL4*, 200 ng *pUAST-GFPken*, 400 ng *pUAST* empty vector, and 1 ng *pUAST-luc* containing the firefly luciferase ORF. Cells were lysed by a freeze-thaw procedure, and luciferase activity was determined to allow for comparison of transfection efficiency.

For reporter assays, 2×10^{7} Kc_{167} cells were transfected with 200 ng of *2x2DrafLuc($STAT^{+}/Ken^{+}$)*, *2x2DrafLuc($STAT^{+}/Ken^{-}$)*, or *2x2DrafLuc($STAT^{-}/Ken^{-}$)* reporters together with 20 ng *pAct-Renilla* and 250 ng *pAc5.1A* empty vector (Invitrogen). For pathway stimulation, cells were cotransfected with 150 ng *pAct-updGFP*. 10 ng *pAct-kenGFP* was cotransfected as appropriate with a commensurate decrease in empty vector. After 12 hr, cells were resuspended in fresh medium and 5×10^{5} cells were seeded into six wells of a 96-well plate. After 72 hr, cells were lysed and measured for *Renilla* and firefly luciferase activities [17]. Values were normalized based on Renilla luciferase activity and are shown as multiples of the unstimulated level. Error bars represent standard deviation, while p values are derived from a Student's t test.

EMSA

To prepare a probe containing STAT92E consensus, the top-strand GGATTTTTCCCGGAAATG and the bottom-strand GACCATTTCCG GGAAAAA oligonucleotides were annealed to give 3′ overhangs. Similarly, oligonucleotides for Ken binding were designed as a palindromic sequence containing inverted repeats of the consensus obtained by SELEX. All probes were labeled with [^{32}P]dCTP via Klenow fragment (Roche). 5 pmols of each probe and 15 μg of total protein were allowed to bind in SELEX binding buffer for 20 min at room temperature. Supershift experiments used a 1:3000 dilution of anti-GFP (Abcam). Complexes were resolved by nondenaturing 4% polyacrylamide gel electrophoresis in 0.5 × TB (44.5 mM Tris-base, 44.5 mM boric acid [pH 9.0]).

Supplemental Data

Supplemental Data include one figure, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://www.current-biology.com/cgi/content/full/16/1/80/DC1/.

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