Developmental Biology
Immunology and Inflammation

Inhibition of Notch activity by phosphorylation of CSL in response to parasitization in *Drosophila*

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Abstract

Notch signaling activity regulates hematopoiesis in *Drosophila* and vertebrates alike. Parasitoid wasp infestation of *Drosophila* larvae, however, requires a rapid downregulation of Notch activity to allow the formation of encapsulation-active blood cells. Here we show that the *Drosophila* CSL transcription factor Suppressor of Hairless [Su(H)] is phosphorylated at Serine 269 in response to parasitoid wasp infestation. As this phosphorylation interferes with the DNA-binding of Su(H), it reversibly inhibits Notch activity. Accordingly, phospho-deficient $Su(H)^{S269A}$ mutants are immune compromised. A screen for kinases involved in Su(H) phosphorylation identified Pkc53E, required for normal hematopoiesis as well as for parasitoid immune response. Genetic and molecular interactions support the specificity of the Su(H)-Pkc53E relationship. Moreover, phorbol ester treatment inhibits Su(H) activity in vivo and in human cell culture. We conclude that Pkc53E targets Su(H) during parasitic wasp infestation, inducing downregulation of Notch activity, thereby remodeling the blood cell population required for wasp egg encapsulation.

eLife assessment

The study by Deichsel et al. reports **valuable** findings that suggest a new, possibly conserved, mechanism by which post-translational modification of a Notch regulator mediates the cellular immune response. However, the claims are only partially supported as the data and analysis are **incomplete**. The work will be of interest to biologists working on immune cell development or regulation of Notch.

Introduction

Drosophila melanogaster harbours a sophisticated cellular immune system to fight invaders. The larval hematopoietic system comprises a circulating and sessile compartment of embryonic origin and the developing larval lymph gland, constituting a hematopietic organ. The circulating and sessile compartment consists primarily of macrophage-like plasmatocytes, plus a small number of crystal cells involved in wound healing and melanization responses to neutralize pathogens. Both cell types differentiate from hemocyte precursors within the lymph gland as well; they are

released during pupal stages to serve the adult with immune cells. Finally, lamellocytes represent the third blood cell type in *Drosophila*. While merely absent in healthy animals, their formation is induced within hours of wasp infestation or wounding (reviewed in: Banerjee et al., 2019; Letourneau et al., 2016; Hultmark and Andó, 2022). In fact, parasitism by endo-parasitoid wasps represents one of the most severe naturally occurring immune challenges to *Drosophila*, invariably causing death if not defended off properly. Parasitoid wasps deposit their egg into the live *Drosophila* larva, where it develops into the next wasp generation egressing from the pupa instead of a fly. Hence, wasp infestation is a life-threatening challenge to the *Drosophila* host, demanding an immediate immune response to overwhelm the parasite. This involves a massive increase in circulating hemocytes, and most energy resources are devoted to fight the invader. Here, lamellocytes play a critical role: they encapsulate the wasp egg and, by expressing Prophenoloxidase, induce a melanization reaction retarding further wasp development (Dudzic et al. 2015; reviewed in: Banerjee et al., 2019; Letourneau et al., 2016; Hultmark and Andó, 2022).

Wasp infestation dramatically remodels the composition of the *Drosophila* hemocyte population (Cho et al., 2020; Tattikota et al., 2020; reviewed in: Csordás et al., 2021). There is a vast increase in plasmatocytes and intermediate precursors in both hematopoietic compartments, from which lamellocyte differentiate, requiring the combined activity of several pathways, including JAK/STAT, JNK, Toll, Notch, EGFR and INR pathways, which in fact regulate blood cell homeostasis in general (reviewed in Banerjee et al., 2019; Letournau et al., 2018; Csordás et al., 2021). Simultaneous to the massive expansion of lamellocytes, crystal cells derived from the same precursors are significantly reduced (Crozatier et al., 2004; Krzemien et al., 2010; Ferguson and Martinez –Agosto, 2014; Cho et al., 2020; Tattikota et al., 2020; reviewed in Csordás et al., 2021). Crystal cells are generated both in the larval lymph gland as well as by trans-differentiation from plasmatocytes in the sessile compartment. Their formation, differentiation and survival strictly depend on Notch signaling activity (reviewed in: Banerjee et al., 2019; Csordás et al., 2021; Hultmark and Andó, 2022). Lamellocyte and crystal cell lineages are mutually exclusive. Accordingly, while promoting crystal cell fate, Notch activity inhibits differentiation of lamellocytes within the lymph gland, however, is reduced upon wasp infestation (Small et al., 2014). Lamellocyte induction involves the formation and sensing of reactive oxygen species triggered by wasp egg injection (Nappi et al., 1995; Sinenko et al., 2011; Small et al., 2014; Louradour et al., 2017; reviewed in: Banerjee et al., 2019; Csordás et al., 2021). The molecular mechanism underlying the simultaneous crystal cell fate inhibition, however, is less well understood. Obviously, an effective and rapid downregulation of Notch activity is required to fend off parasitic wasps.

The Notch pathway is highly conserved between invertebrates and vertebrates, where it regulates numerous cell fate decisions. Notch signals are transduced by CSL type proteins (abbreviation of human CBF1/RBPJ, *Drosophila* Suppressor of Hairless [Su(H)], and worm Lag1), that bind the DNA to direct transcriptional activity of Notch target genes by help of recruited cofactors (reviewed in: Giaimo et al., 2021; Kopan and Ilagan, 2009; Siebel and Lendahl, 2017). Hence, CSL is central to Notch pathway activity as no signal transduction could occur in its absence or in the instance of a lack of DNA-binding. Earlier, we observed CSL, i.e. Su(H) phosphorylation at Serine 269 in cultured *Drosophila* Schneider S2 cells (Nagel et al., 2017). Of note, Schneider S2 cells have hemocyte characteristics (Cherbas et al., 2011; Schneider, 1972; Terriente-Felix et al., 2013). As a consequence of the negative charge conferred by the phosphorylation at Serine 269, Su(H) loses its affinity to the DNA, and without its DNA-binding ability, also its function as a transcriptional regulator (Nagel et al., 2017). Accordingly, a phospho-mimetic $Su(H)^{S269D}$ mutant behaved like a *Su(H)* loss of function mutant in all respects. In contrast, the phospho-deficient $Su(H)^{S269A}$ variant, appeared wild type at the first glance, indicating some tissue-specificity of this regulatory mechanism. In fact, the $Su(H)^{S269A}$ allele displayed a gain of Notch activity particularly during embryonic and larval hematopoiesis with increased numbers of crystal cells in both hematopoietic compartments (Frankenreiter et al., 2021). Moreover, we found that the general

Notch antagonist Hairless is not involved in constraining crystal cell numbers, suggesting that in the context of blood cell homeostasis Notch activity is regulated by the phosphorylation of Su(H) (Maier, 2006; Frankenreiter et al., 2021). As the same set of genes affect blood cell maintenance and differentiation in homeostasis and upon immune challenge (Cho et al., 2020; Tattikota et al., 2020), obviously phospho-mediated downregulation of Notch activity might also occur in response to parasitoid wasp infestation, allowing the formation of encapsulation active lamellocytes at the expense of crystal cells. Briefly, phosphorylation of Su(H) is an elegant mechanism to promptly and transiently curb Notch activity.

In this work, we followed the tempting hypothesis that following wasp infestation, a specific kinase might be activated to phosphorylate Su(H) thereby blocking Notch activity. In this case, the phospho-deficient $Su(H)^{S269A}$ allele should be immune-compromised, as it cannot respond to phosphorylation, i.e. remaining active even upon infection. Indeed, $Su(H)^{S269A}$ displayed an increased sensitivity towards parasitoid wasp infestation accompanied by an increase of crystal cells at the expense of lamellocytes. Accordingly, phosphorylation of Su(H) protein was detected in infested wild type larvae but not in the $Su(H)^{S269A}$ mutant. In a screen for kinases regulating this process, Pkc53E, the homologue of human PKCα, was identified as an important player. In agreement with a role in blood cell homeostasis, a $Pkc53E^{\Delta 28}$ null mutant displayed increased crystal cell numbers. Moreover, genetic and molecular interactions between Su(H) and Pkc53E support the specificity of their relationship. Finally, $Pkc53E^{\Delta 28}$ was impaired in its immune response to wasp infestation as well. Together, these data show that Su(H) is a target of Pkc53E during parasitic wasp infestation, inducing phosphorylation and subsequent downregulation of Notch activity to allow the mass production of lamellocytes required for wasp defense.

Results

Impaired immune response to parasitoid wasps in the phospho-deficient $Su(H)^{S269A}$ mutant

Wasp infestation alters the course of hematopoiesis, as lamellocyte differentiation is massively increased at the expense of crystal cells. This process requires a rapid downregulation of Notch activity that may be implemented by the phosphorylation of Su(H) at Serine 269 (S269). To test this model, we exposed control larvae and larvae of the phospho-deficient $Su(H)^{S269A}$ variant to the parasitoid wasp *Leptopilina boulardi* (*L. boulardi*) and analysed the consequences on blood cell homeostasis two days post-infection. To exclude any influence of the engineered genomic background, we used $Su(H)^{gwt}$ for the comparison, carrying a genomic wild type construct in place of the mutant (Praxenthaler et al., 2017; Frankenreiter et al., 2021).

Earlier we noted an excess of crystal cells in the phospho-deficient $Su(H)^{S269A}$ allele, which we interpret as a gain of Notch activity in consequence of the inability to be downregulated by Su(H) phosphorylation (Frankenreiter et al., 2021) (**Fig. 1A,B**). In response to wasp infestations, however, crystal cell numbers should drop to allow formation of lamellocytes (Small et al., 2014; Csordás et al., 2021). Indeed in the $Su(H)^{gwt}$ control, both the sessile crystal cells as well as those within the larval lymph glands were significantly lessened in response to wasp infestation (**Fig. 1A,B**). In contrast, the higher crystal cell numbers in the $Su(H)^{S269A}$ mutant larvae dropped to control-level only, demonstrating the inability of the mutant to detect this immune challenge or to respond to it (**Fig. 1A,B**).

Increased abundance of lamellocytes upon wasp infestation was monitored *in vivo* in larval hemolymph and lymph glands, using either the L1-*atilla*-GFP reporter (Honti et al., 2009) or *PPO3*-Gal4::UAS-GFP (Dudzic et al., 2015), respectively. In the harvested larval hemolymph of the infested $Su(H)^{gwt}$ control, the hemocytes contained about 15% *PPO3*- and 23% *atilla*-labelled

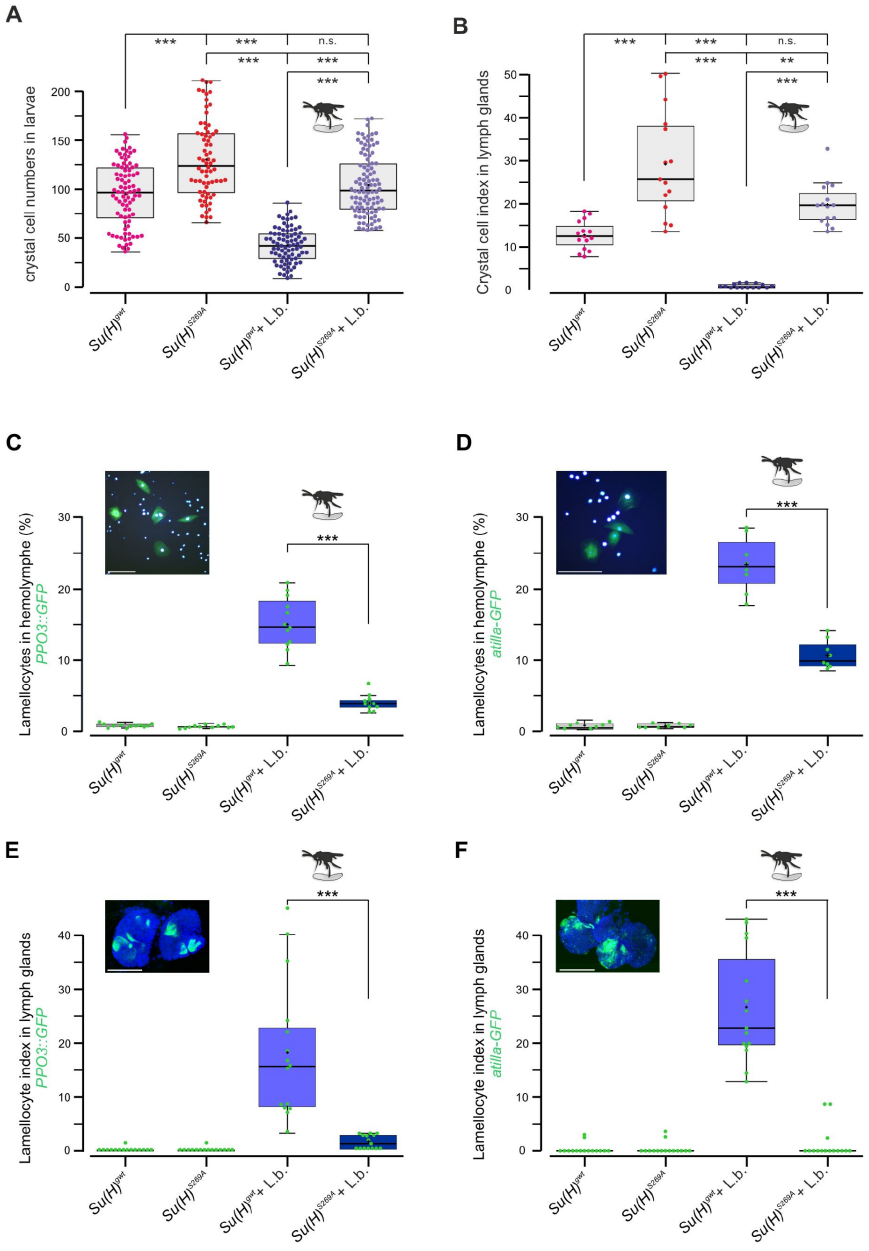

Figure 1.

Su(H)^{S269A} mutants are compromised in their response to parasitoid wasp infestation

(**A**) Quantification of melanized crystal cells from the last two segments of *Su(H)^{gwt}* and *Su(H)^{S269A}* larvae with and without wasp infestation as indicated. In the control, wasp parasitism causes crystal cell numbers to drop to a level of about 50%, whereas in *Su(H)^{S269A}* mutants the number settles at the un-infested *Su(H)^{gwt}* level. Each dot represents one analysed larva (n=70-100). (**B**) Crystal cell index in larval lymph glands is given as ratio of Hnt-positive crystal cells per 1° lobe relative to the size of the lobe. Each point represents one analysed lobus (n=15). Statistical analyses with ANOVA for multiple comparisons, using Tukey-Kramer approach with *** p≤0.001, ns (not significant p>0.05).

(C-F) Quantification of larval lamellocytes in the circulating hemolymph (C,D) or in lymph glands (E,F) before and after wasp infestation in *Su(H)^{gwt}* versus *Su(H)^{S269A}*. Lamellocytes were marked with either *PPO3-Gal4::UAS*-GFP (C,E) or *atilla*-GFP (D,F) as indicated. (C,D) The fraction of GFP-labelled lamellocytes of the total number of DAPI-labelled blood cells isolated from hemolymph is given; each dot represents ten pooled larvae. Representative image of labelled control hemolymph is shown above (DAPI-labelled nuclei in light blue, GFP in green). Scale bars 50 µm.

(E,F) Lamellocyte index is given as number of GFP-labelled lamellocytes per area in the 1° lobe of the lymph gland. Each dot represents the lamellocyte index of one lobus (n=12).

Representative *Su(H)^{gwt}* lymph glands after infestation are shown above, co-stained for nuclear Pzg (in blue). Scale bars 100 µm. Statistical analyses with ANOVA using Dunnet's approach relative to *Su(H)^{gwt}* control; only significant differences are indicated (*** p≤0.001).

lamellocytes (**Fig. 1C,D**). These numbers were significantly lower in the wasp infested *Su(H)*^{S269A} larvae (Fig. C,D). Consistently, both lamellocyte reporters were robustly induced in the lymph glands of the infested control, but rarely in glands of the *Su(H)*^{S269A}mutant (**Fig. 1E,F**). Obviously, the *Su(H)*^{S269A} mutant barely responds to the immune challenge raised by the parasitic wasp infestation. Overall, these data support the model that S269 in Su(H) is a molecular target for a kinase, phosphorylated upon immune challenge to downregulate Notch activity, thereby allowing lamellocyte formation at the expense of crystal cells.

The phospho-deficient Su(H)^{S269A} allele cannot combat wasp infestation

Parasitoid wasp infestation constitutes an extreme immune challenge for the *Drosophila* larva: if not combatted by the immune system, a wasp egg, which is deposited in the larval body cavity, will develop into an adult wasp, thereby killing the larval host during pupal stage. Indeed, depending on the wasp species used, we measured a high mortality rate with less than 5% up to about 15% of surviving flies (**Fig. 2A**). According to our working hypothesis, the phospho-deficient *Su(H)*^{S269A} variant should not be able to properly respond to the immune challenge. To test this directly, we measured the survival of *Su(H)*^{S269A} animals upon wasp infestation compared to the wild type control.

The two closely related wasp species *L. boulardi* and *Leptopilina heterotoma* (*L. heterotoma*) very efficiently parasitized both the control *Su(H)*^{gwt} and the *Su(H)*^{S269A} variant, though the latter appeared slightly more sensitive (**Fig. 2A**). The difference in mortality became more apparent with the wasp species *Asobara japonica* (*A. japonica*), allowing 14.5% of the *Su(H)*^{gwt} control flies to escape parasitism, whereas only 3.8% of the infested *Su(H)*^{S269A} pupae emerged as flies. Thus, the *Su(H)*^{S269A} mutants are less robust in resisting parasitoid wasp infestation consistent with a defective immune response.

Serine 269 of Su(H) is phosphorylated upon wasp infestation

Next, we wanted to directly monitor Su(H) phosphorylation at S269 in response to wasp infestation. To this end, polyclonal antibodies directed against a phosphorylated peptide containing the sequence motif NRLRpSQTVSTRYLHVE were generated (α-pS269). Specificity was first tested by Western blot analysis using bacterially expressed GST fusion proteins containing the entire beta-trefoil domain (BTD) of Su(H), as well as with phospho-mimetic (S269D) and phospho-mutant (S269A) versions. All three variants were detected by the antisera. The S269D version, however, was strongly preferred, indicating that this antibody does preferably recognize phospho-S269 Su(H) protein (**Fig. 2** - figure supplement 1). Encouraged by this result, we used this antiserum on protein extracts derived from larvae infested and not infested by *L. boudardi*. For this experiment we used genome engineered fly strains expressing mCherry-tagged Su(H) proteins, *Su(H)*$^{S269A\text{-}mCh}$and *Su(H)*$^{gwt\text{-}mCh}$for control (Praxenthaler et al., 2017). Su(H) proteins were trapped using RFP-nanobody coupled agarose beads, and the precipitates were then probed in Western blots with antibodies directed against mCherry and pS269. Indeed, α-pS269 antibodies recognized Su(H)$^{gwt\text{-}mCh}$ protein specifically in wasp infested larvae, indicating respective phosphorylation of Su(H) protein. No such signals were seen in precipitates from the non-infected larvae nor from any of the *Su(H)*$^{S269A\text{-}mCh}$ mutant larvae (**Fig. 2B**). These data clearly show that wasp infestation induced the phosphorylation of Su(H) at S269. Apparently, parasitoid wasp infestation starts a cascade of events resulting in the inhibitory S269 phosphorylation of Su(H) protein to promptly interrupt Notch signalling activity and to allow lamellocyte formation. Hence, the question arose on the kinase/s involved in this process.

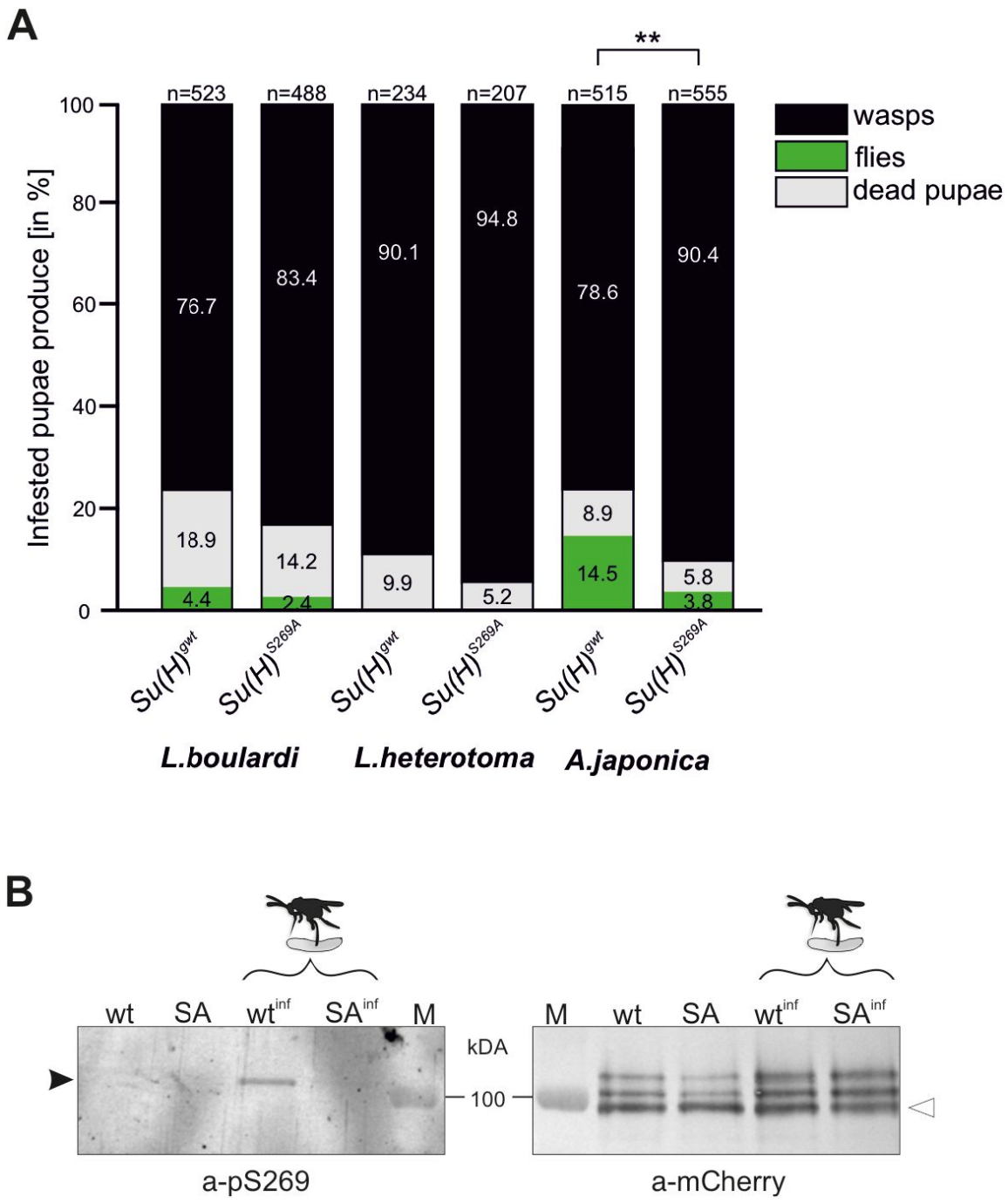

Figure 2.

Resistance to wasp infestation and phosphorylation at S269

(A) Resistance of *Su(H)*^{gwt} and *Su(H)*^{S269A} to the infestation with parasitic wasp strains *L. boulardi* and *L. heterotoma* (both family Figitidae) and *A. japonica* (family Braconidae), as indicated. Numbers of eclosed flies versus wasps as well as of dead pupae are presented in relation to the total of infested pupae. At least three independent experiments were performed, n=number of infested pupae. Statistically significant difference determined by Student's T-test is indicated with **p≤0.01.

(B) Su(H) is phosphorylated at Serine 269 upon parasitoid wasp infestation. Protein extracts from *Su(H)*$^{gwt\text{-}mCh}$ (wt) and *Su(H)*$^{S269A\text{-}mCh}$ (SA) larvae, respectively, infested (wt^{inf}, SA^{inf}) or not with *L. boulardi*, were isolated by RFP-Trap precipitation and probed in Western blots. The α-pS269 antiserum specifically detects wild type Su(H) protein only in wasp infested larvae (arrowhead), but not the Su(H)^{S269A} isoform. The blot on the right serves as loading control, probed with α-mCherry antibodies, revealing the typical Su(H) protein pattern in all lanes; the lowest band presumably stems from degradation (open arrowhead). M, prestained protein ladder, protein size is given in kDa.

Screening of kinase candidates mediating phosphorylation of Su(H) at Serine 269

To identify Ser/Thr kinases involved in the phosphorylation of Su(H) at S269, we commenced with a combination of *in silico* and biochemical approaches aiming to generate a list of candidate kinases which can be further analysed by genetic means (**Fig. 3**). First, by using GPS 3.0 software that encompasses a substantial database of kinases and their preferred recognition motives (Xue et al., 2011), 36 potential human kinases were predicted to recognize S269 as substrate, represented by 30 kinases in *Drosophila* (**Fig.3** – supplement Table 1). In addition, the BTD domain of Su(H) was bacterially expressed as a GST fusion protein and subjected to phospho-assays using 245 different human Ser/Thr kinases. Our reasoning for using the entire BTD domain was to ensure a normal folding of the domain (Kovall and Blacklow, 2010), and to reduce the number of potential phospho-sites at the same time present in full length Su(H). With this approach, we ended up with 62 human Ser/Thr kinases (25% of the tested kinases) that use the BTD of Su(H) as an *in vitro* substrate. These kinases correspond to 40 different kinases in *Drosophila* belonging to six different kinase families (**Fig.3** –supplement Table 2). Ten of these kinases were also predicted *in silico*, representing members of the AGC, CAMK, CMGC, STE and OPK family of kinases (**Fig.3** –supplement Table 2). Both sets of data, biochemical and bioinformatics, were used to generate a list of 44 candidates to be analysed by genetic means (**Fig.3** –supplement Table 3). The candidates were further screened for an imbalanced hematopoiesis. We reasoned that mutants affecting a relevant kinase gene involved in the phosphorylation of Su(H) should display increased crystal cell numbers similar to what was observed in the $Su(H)^{S269A}$ mutant (Frankenreiter et al., 2021). Larvae of 13 different kinase mutants and the progeny of 44 UAS-RNAi and/or UAS-kinase dead transgenes crossed with *hml*-Gal4, a blood cell-specific Gal4 driver line, were tested (**Fig.3** –supplement Table 3). To this end, the larvae were subjected to heating. This procedure allows a comfortable visualization and quantification of mature larval crystal cells through the cuticle, which blacken due to the activation of Prophenoloxidase (Rizki, 1957; Lanot et al., 2001). Fourteen kinase mutants tested were similar to the control, whereas mutations in four kinases impeded crystal cell development or prevented it altogether. Unexpectedly, the majority of the tested kinase mutants exhibited elevated crystal cell numbers, however to a different degree (**Fig.3** –supplement Table 4). Seven kinase mutants substantially exceeded crystal cell numbers seen in $Su(H)^{S269A}$ larvae; they were hence excluded from further analysis. Nineteen kinase mutants matched closely the $Su(H)^{S269A}$ phenotype, making those the most promising candidates for being involved in the phosphorylation of Su(H) at S^{269}. Six of those were within the cluster of ten candidates singled out by the *in silico* and the *in vitro* screens (**Fig. 3** A,B). Using commercially available, activated human kinases, we were able to test five candidates, AKT1, CAMK2D, GSK3B, S6 and PKCα in vitro by MS/MS analysis on the Su(H) peptide ALFNRLR**S**^{8}QTVSTRY, where Serine 8 corresponds to S269 in Su(H). Only PKCα unambiguously phosphorylated the given peptide at Serine 8. Whereas GSK3B did not phosphorylate the peptide at all, AKT1, CAMK2D and S6 piloted Threonine 10, corresponding to Threonine 271 in Su(H) (**Fig. 3D** -figure supplement 1). Together, these data support the idea, that PKCα corresponding to Pkc53E in *Drosophila*, is part of the kinase network mediating the phosphorylation of Su(H) at S269.

Role of Pkc53E in the phosphorylation of $Su(H)^{S269}$

Confirming the MS/MS data, human PKCα was able to phosphorylate the respective Su(H) peptide (S^{wt}) similar to its defined pseudo-substrate PS (Kochs et al., 1993), whereas the S8A mutant peptide (S^{SA}) was accepted only half as well in an ADP-Glo^{TM} assay, indicating that S269 is a preferred substrate (**Fig. 4A**). Bacterially expressed and purified *Drosophila* Pkc53E, however, did neither accept the PS nor the Su(H) peptides (**Fig. 4B**). Pkc53E activity, however, was stimulated by the agonistic phorbol ester PMA (phorbol 12-myristate 13-acetate) (Blumberg et al., 1983; Nakashima, 2002) in phosphorylating the PS and Su(H) peptide S^{wt} but not the S8A mutant peptide S^{SA} (**Fig. 4C**). To generate an activated form of Pkc53E, we exchanged four

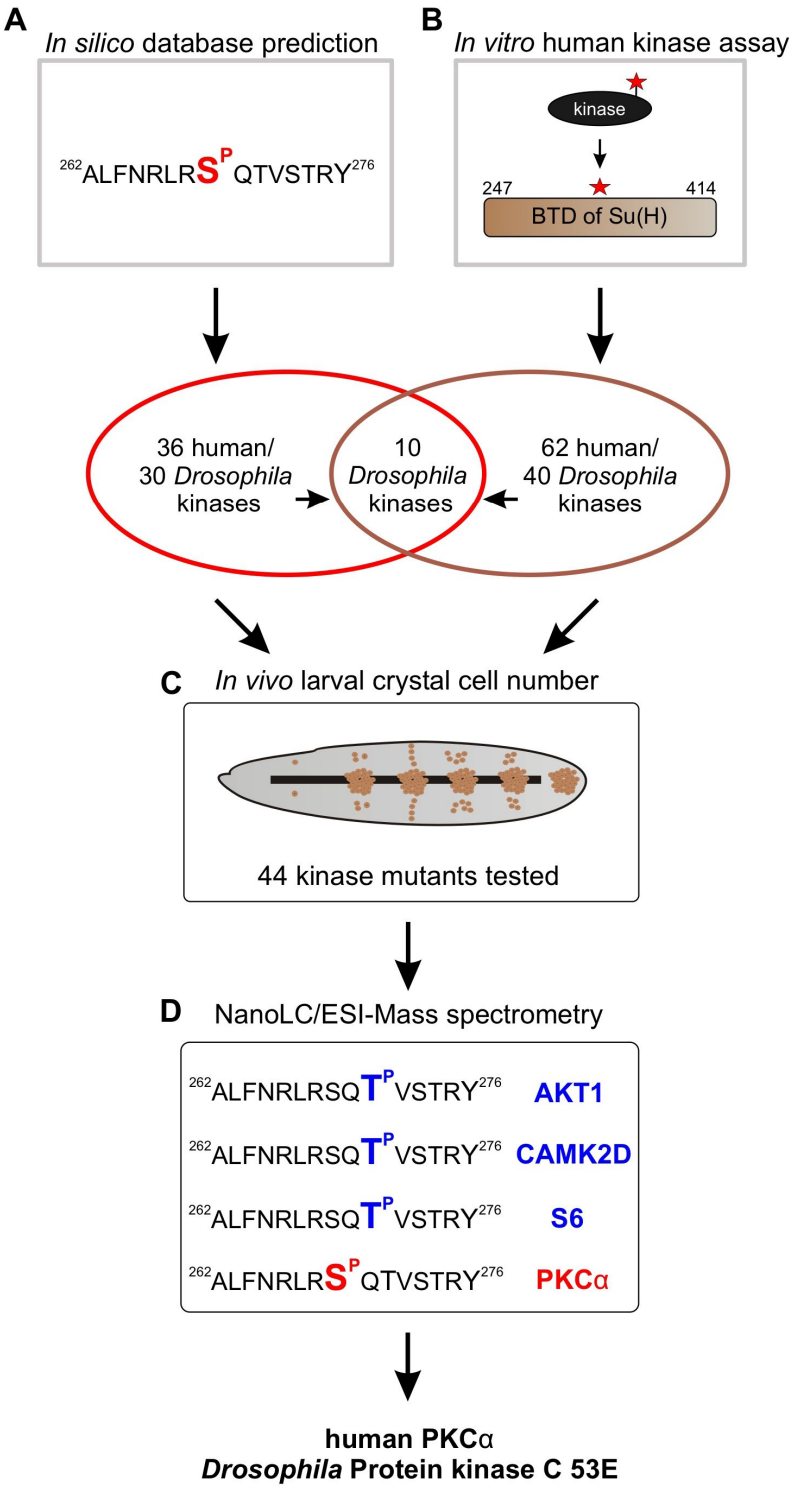

Figure 3.

Pipeline of screening procedures for kinase candidates triggering phosphorylation at Su(H)^{S269}

(A) *In silico* screening of database(s) predicting kinase recognition motif in Su(H)^{S269}; see supplement Table 1. **(B)** *In vitro* assay screening 245 human Ser/Thr kinases for their ability to phosphorylate the BTD domain of Su(H); see supplement Table 2. **(C)** *In vivo* screen of 44 different *Drosophila* kinase mutants for crystal cell occurrence in third instar larvae; see supplement Table 4. **(D)** NanoLC/ESI mass spectrometry with active human kinases monitoring their ability to phosphorylate the given Su(H) peptide. PKCα phosphorylates S269, whereas AKT1, CAMK2D and S6 kinase prefer T271. Spectra are shown in figure supplement 1.

codons by *in vitro* mutagenesis, three (T508D, T650D and S669D) mimicking phosphorylation in the kinase and C-terminal domains, respectively, and one in the pseudo-substrate domain (A34E) (**Fig.4** – figure supplement 1) (Gould and Newton, 2008). The resultant Pkc53E^{EDDD} protein accepted the PS, and the Su(H) peptide S^{wt} even better, but not the S8A mutant peptide S^{SA}, demonstrating the specificity of the phosphorylation (**Fig. 4D**). As predicted for a fully activated kinase, PMA was unable to boost Pkc53E^{EDDD} protein activity any further (**Fig. 4E**).

The PKC-agonist PMA influences Su(H) activity and blood cell homeostasis

Our data so far indicated that Su(H) is a phospho-target of Pkc53E which reduces its activity by affecting its DNA-binding. In this case, we might expect an influence of the general PKC-activator PMA on both, Su(H) activity as well as Notch-mediated crystal cell formation. We tested the former in a *RBPJ^{ko}* HeLa cell system (Wolf et al., 2019), measuring Notch-reporter gene activation by Su(H)-VP16, as this Su(H) variant is independent of Notch activity itself, allowing to directly monitor the influence of PMA on Su(H) activity. Indeed, Su(H)-VP16 ability to activate reporter gene transcription was reduced by more than half in the presence of PMA (**Fig. 5A**). This is in agreement with an PMA-mediated activation of endogenous PKC in the transfected HeLa cells, resulting in Su(H)-VP16 phosphorylation, loss of DNA-binding activity and reduced transcriptional activation. Accordingly, repression could be reverted by the addition of kinase-inhibitor Staurosporine (Karaman et al., 2008). In fact, Staurosporine alone already increased Su(H)-VP16 transcriptional activity, indicating that PKC-driven phosphorylation occurs to a considerable degree in the normal situation in HeLa cells already (**Fig. 5A**).

Next, we assayed the effect of PMA on larval crystal cell formation. If, as expected, PMA increased Pkc53E activity, Su(H) should be inactivated by phosphorylation with decreased crystal cell numbers as a consequence. We fed PMA to *Drosophila* larvae and assayed the numbers of sessile crystal cells. Indeed, crystal cell numbers dropped to nearly zero, similar of what is observed in the phospho-mimetic *Su(H)^{S269D}*mutant (Frankenreiter et al., 2021), suggesting a very efficient phosphorylation and inactivation of Su(H) protein (**Fig. 5B**). In contrast, *Su(H)^{S269A}* mutant larvae displayed an increased number of crystal cells which can be attributed to the fact that here, Su(H) can no longer be phosphorylated and hence, is overactive in this context. Feeding PMA to *Su(H)^{S269A}* larvae only caused a drop of excessive crystal cell numbers down to a wild type level (**Fig. 5B**). In conclusion these data show that Su(H) activity is regulated *in vitro* and *in vivo* by PKC activity in the context of blood cell homeostasis.

Pkc53E is required for normal blood cell homeostasis in Drosophila larvae

Su(H)^{S269A} mutant larvae develop an excess of crystal cells, both in the hemolymph as well as in the lymph glands, due to a failure to downregulate respective Notch activity in the hemocyte precursors via the phosphorylation of Su(H) protein (Frankenreiter et al., 2021) (see **Fig. 1A**). Assuming Pkc53E has a major role in the phosphorylation of Su(H), we should expect a similar phenotype in a *Pkc53E* mutant due to the inability to phosphorylate any substrate. In order to test this assumption directly, we assessed the number of crystal cells in larval lymph glands as well as in sessile crystal cells in several loss of function backgrounds of *Pkc53E*. To this end, we used the *Pkc53E$^{\Delta 28}$* allele, which by RT-PCR is a null mutation (**Fig. 6** – figure supplement 1). In addition, we used two different RNAi-lines and one sgRNA line under UAS-control to knock down *Pkc53E* activity specifically within the developing lymph gland using *lz*-Gal4 and within the hemocytes using *hml*-Gal4, respectively (Lebestky et al., 2000). In any context tested, the number of crystal cells was strongly increased matching those of the *Su(H)^{S269A}* mutant (**Fig. 6**). The similar phenotypes imply that Pkc53E acts through the phosphorylation of Su(H). However, as outlined above, the majority of kinase mutants displayed increased crystal cell numbers, raising the

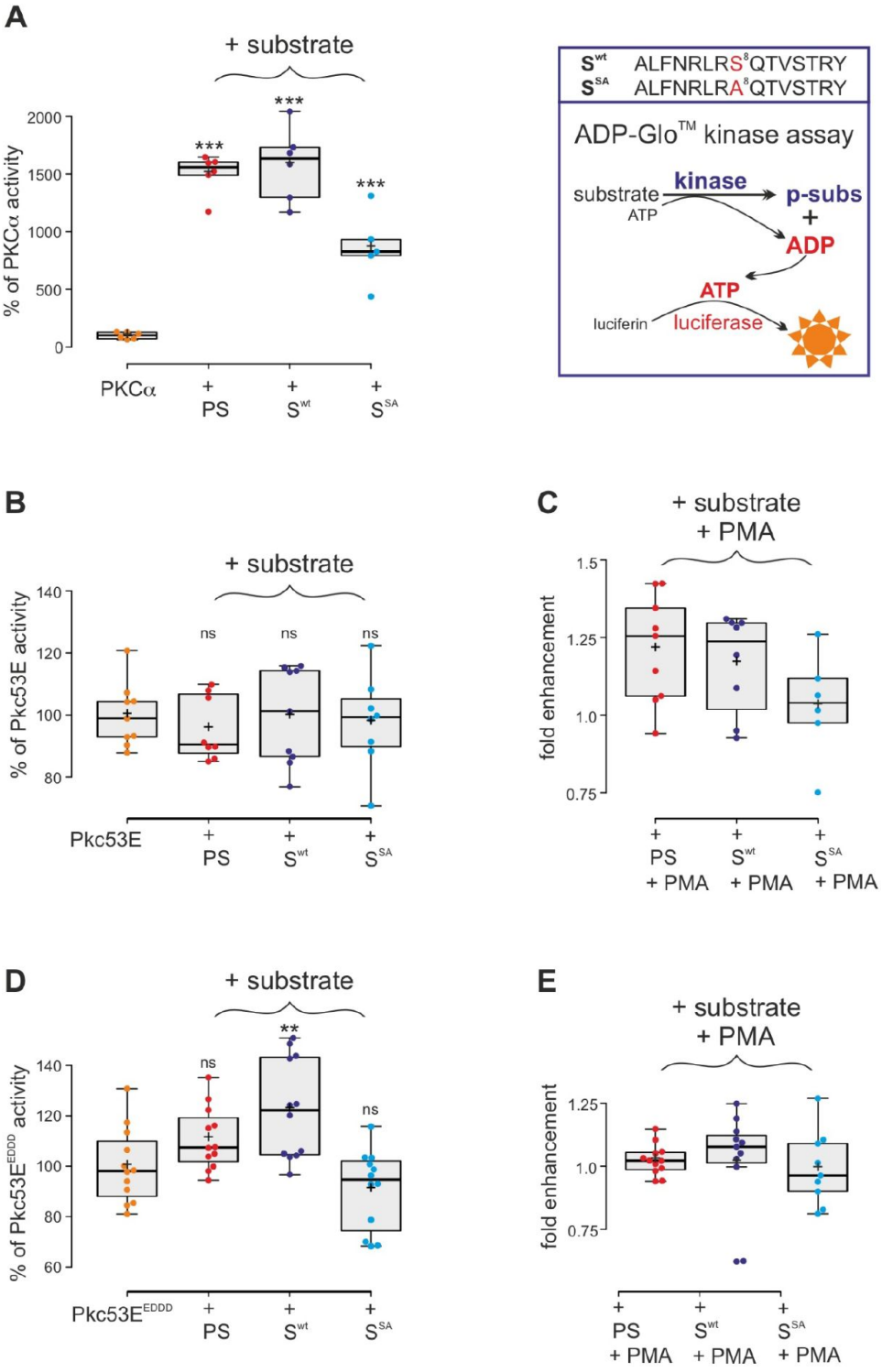

Figure 4.

Kinase assays using activated PKCα and Drosophila Pkc53E variants

(**A**) Right, schema of ADP-Glo^{TM} assay to quantify kinase activity. The wild type (S^{wt}) and mutant (S^{SA}) Su(H) peptides 262-276 offered as specific kinase substrates are indicated above. Left, commercially available, active PKCα very efficiently phosphorylates the pseudo-substrate PS and the Su(H) S^{wt} peptides, but less efficiently the S^{SA} mutant peptide. Activity is given as percentage of the auto-active kinase without substrate. (**B**) Bacterially expressed Pkc53E has no activity on any of the offered substrates PS, S^{wt} or S^{SA}. (**C**) PMA raised Pkc53E activity to nearly 125% for PS and S^{wt} but not for S^{SA}. (**D**) Activated $Pkc53^{EDDD}$ triggers phosphorylation of PS and S^{wt} but not of S^{SA}. (**E**) Addition of PMA does not change $Pkc53^{EDDD}$ activity.

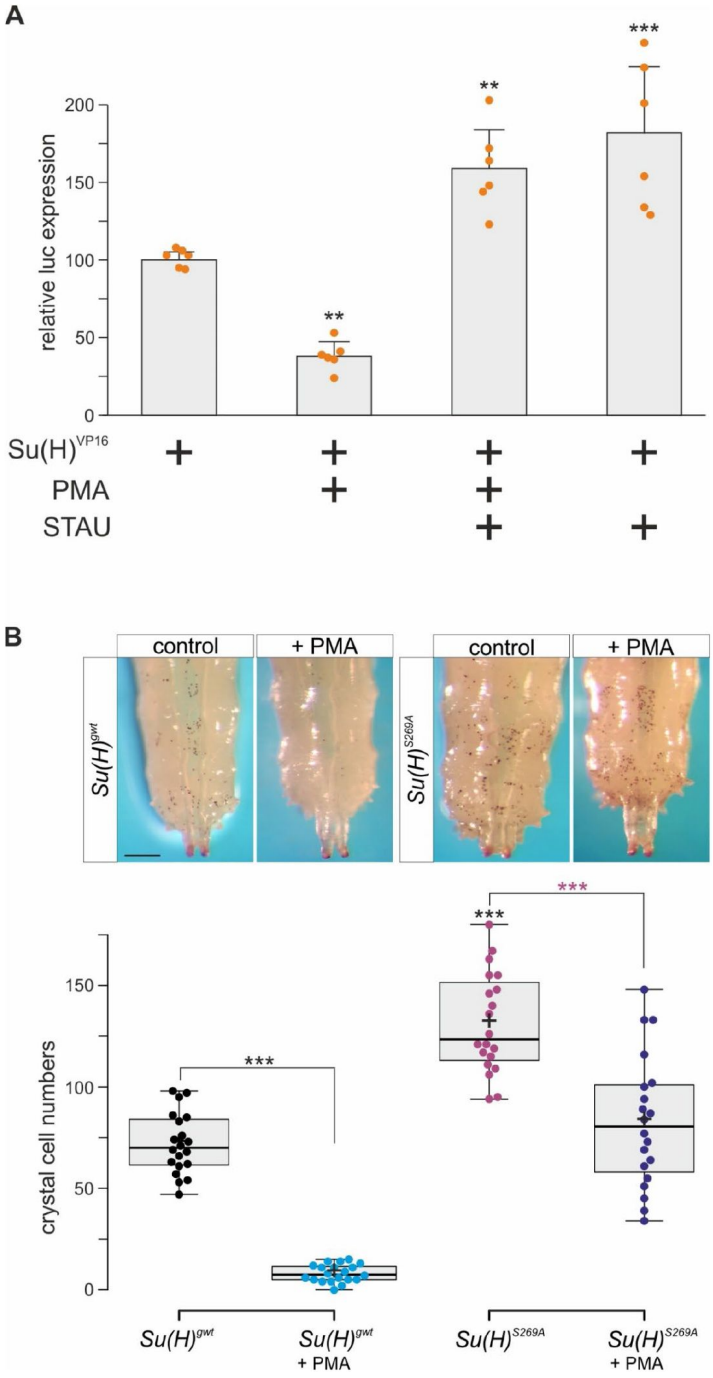

Figure 5.

PMA induced activation of Pkc53E inhibits Su(H) transcriptional activity in vitro and crystal cell formation in vivo

(A) 2xMyc-Su(H)-VP16 (SuH)^{VP16}), transfected into *RBPj^{ko}* HeLa cells, activates expression of a luciferase-reporter gene independent of Notch activity. Luciferase activity is given relative to the value of the reporter construct alone; the Su(H)-VP16 value is taken as 100%. Addition of PMA reduces Su(H)-VP16 dependent transcriptional activity to about 40%, which is reversed by the inhibitor Staurosporine (STAU). STAU itself increases Su(H)-VP16 activity nearly twofold. Six independent experiments were performed. Bars show the mean, error bars indicate standard deviation. Statistical analysis was performed with a two-way ANOVA for multiple comparisons, Dunnet's test with *** p≤0.001, ** p≤0.01 relative to Su(H)-VP16 alone.
(B) Number of melanized larval crystal cells determined in the last two segments of larvae fed with normal fly food, or with fly food plus PMA (n=20). Note strong drop of crystal cell numbers in the *Su(H)^{gwt}* control fed with PMA, in contrast to the *Su(H)^{S269A}* mutant larvae, where numbers drop to only control level. Representative animals are shown above; scale bar 250 µm. Statistical analysis by two-way ANOVA for multiple comparisons, Tukey-Kramer approach (*** p≤0.001), significant differences are colour coded.

possibility of a fortuitous accordance. If the increase of crystal cell numbers in *Pkc53E* mutants is independent of Su(H) phosphorylation, we should expect an additive effect if we combine the two mutants. The double mutants $Su(H)^{S269A}$ $Pkc53E^{\Delta 28}$ were generated by genetic recombination; they displayed the same range of excessive crystal cell numbers as the single mutants (**Fig. 7A,B**). Moreover, the strongly reduced number of crystal cells observed in the overactive $Su(H)^{S269D}$ mutant was not increased by $Pkc53E^{\Delta 28}$ (**Fig. 7A,B**), indicating that Pkc53E indeed acts upstream of Su(H), or directly on Su(H). If the latter is the case, we may expect the two proteins to form complexes *in vivo*. Indeed, we could co-precipitate Su(H)-Pkc53E protein complexes, both from *Drosophila* heads containing hemocytes (Sanchez Bosch et al., 2019), as well as from the larval hemolymph (**Fig. 7C,D**). Specific co-precipitation was eased by using fly strains expressing m-Cherry tagged Su(H) (Praxenthaler et al., 2017) and HA-tagged Pkc53E, respectively. Together, these data demonstrate that *Pkc53E* has an important role in blood cell homeostasis that can be largely explained by its activity to phosphorylate Su(H), thereby regulating Notch activity during hemocyte and lymph gland development.

Pkc53E mutants are immune compromised

According to our hypothesis, Pkc53E phosphorylates Su(H) in response to an immune challenge by parasitic wasp infestation. Hence, we would expect that a loss of Pkc53E function should affect the ability of *Drosophila* larvae to fight wasp infestations similarly to the $Su(H)^{S269A}$ mutant. Indeed, when the $Pkc53E^{\Delta 28}$ null mutant was infested with the parasitic wasp *L. boulardi*, lamellocyte numbers in the hemolymph did not reach wild type levels, and they were absent from the larval lymph glands (**Fig. 7E,F**). Apparently, the $Pkc53E^{\Delta 28}$ null mutant is unable to recognize parasitic wasp infestation or is unable to respond to it, for example by the phosphorylation of Su(H), demonstrating the involvement of this kinase in the immune response of *Drosophila* to parasitoid wasp infestation.

Discussion

The Notch pathway is highly conserved between invertebrates and vertebrates, with regard to both the underlying molecular principles as well as the biological processes it is involved, including hematopoiesis and immune defence. During mammalian hematopoiesis, Notch plays a fundamental role in stem cell maintenance and proliferation as well as in the differentiation of blood cell precursors, notably in T-cell development. Accordingly, aberrant Notch signaling activity has profound consequences for blood cell homeostasis that may result in leukemia (reviewed in: Radtke et al., 2010; Siebel and Lendahl, 2017; Banerjee et al., 2019; Gallenstein et al., 2023). Hence, the principles of the regulation of Notch signaling activity are of great interest.

Notch signals are transduced by CSL-type DNA-binding proteins that are pivotal to Notch pathway activity, as no signal transduction could occur in their absence or in the instance of a lack of DNA-binding. CSL proteins are extremely well conserved. They act as a molecular switch, either activating or repressing Notch target genes, depending on the recruited cofactors. Upon ligand binding, the Notch receptor is cleaved, and the biologically active Notch intracellular domain (NICD) is released, to assemble an activation complex with CSL and further co-factors. Notch pathway repression, however, entails for example the recruitment of co-repressors by CSL; in *Drosophila* mediated by the binding of Hairless (reviewed in: Maier, 2006; Borggrefe and Oswald, 2009; Kovall and Blacklow, 2010; Bray, 2016; Giaimo et al., 2021).

Studies of *Drosophila* hematopoiesis have uncovered a pleiotropic role of Notch in all the hematopoietic compartments, where Notch activity needs to be precisely regulated to ensure blood cell homeostasis (reviewed in: Banerjee et al., 2019; Csordás et al., 2021). During blood

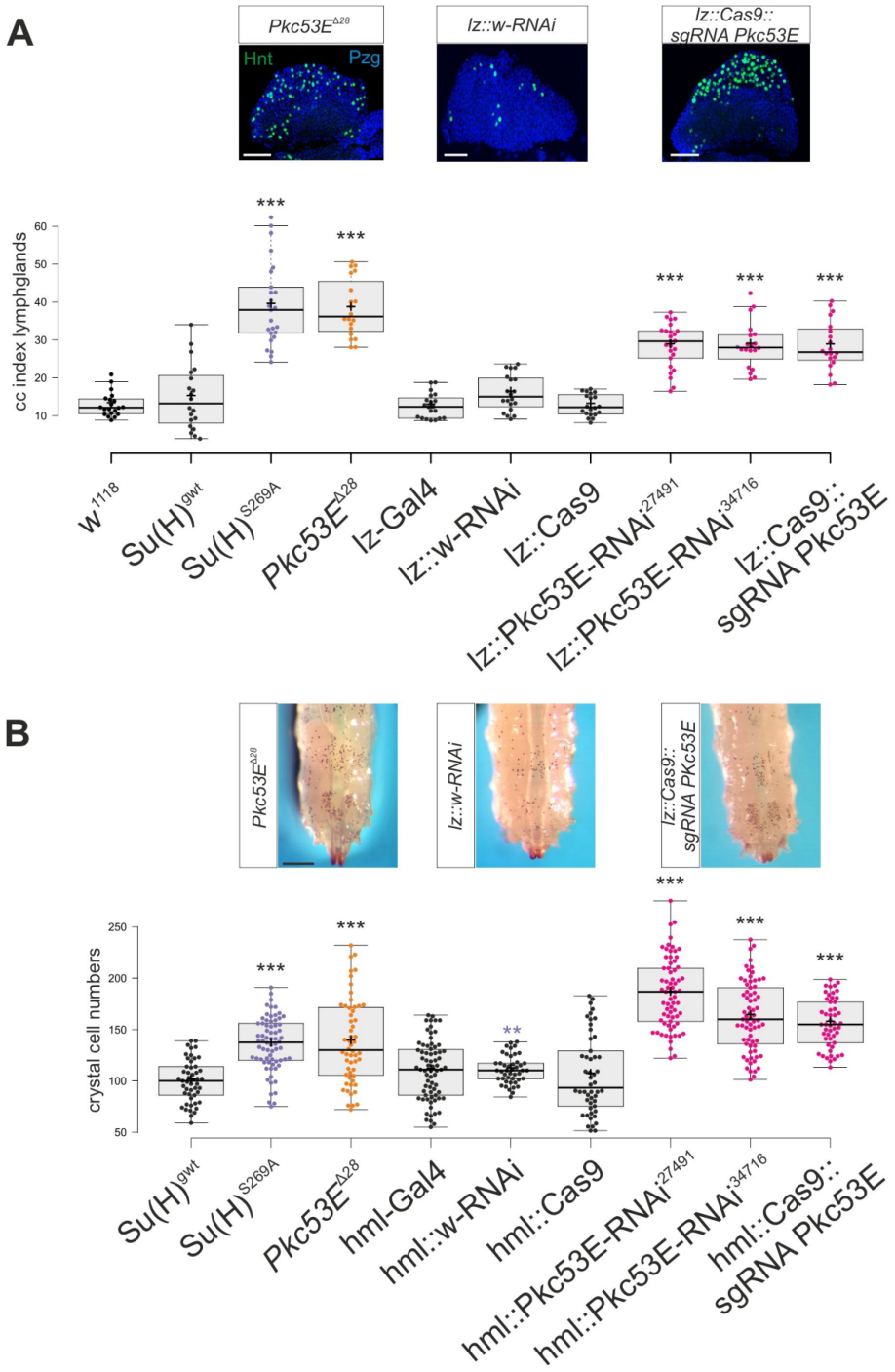

Figure 6.

Loss of Pkc53E causes a gain of crystal cell number

Depletion of *Pkc53E* activity in the *Pkc53E*$^{\Delta 28}$ mutant or after knock down by *Pkc53E*-RNAi or sg*Pkc53E* with the help of the Gal4/UAS system using *lz*-Gal4 (A) or *hml*-Gal4 (B).

Controls as indicated. **(A)** Crystal cell index in lymph glands; each dot represents the value of an analysed lobus (n= 20-25). Representative examples of *Pkc53E*$^{\Delta 28}$, *lz*::*Pkc53-RNAi* and *lz*::*Cas9 sgPkc53E* are shown above. Crystal cells are labelled with Hnt (green), the lobe is stained with α-Pzg (blue). Scale bar 50 µm.

(B) Melanized crystal cells enumerated from the last two segments of larvae with the given genotype (n=45-70). Representative examples of the evaluated last segments of *Pkc53E*$^{\Delta 28}$, *lz*::*Pkc53*-RNAi and *lz*::*Cas9 sgPkc53E* are shown above. Scale bar 250 µm. ANOVA for multiple comparisons, Dunnet's approach relative to controls with *** p≤0.001. Note that there were no significant differences between any of the controls shown in black.

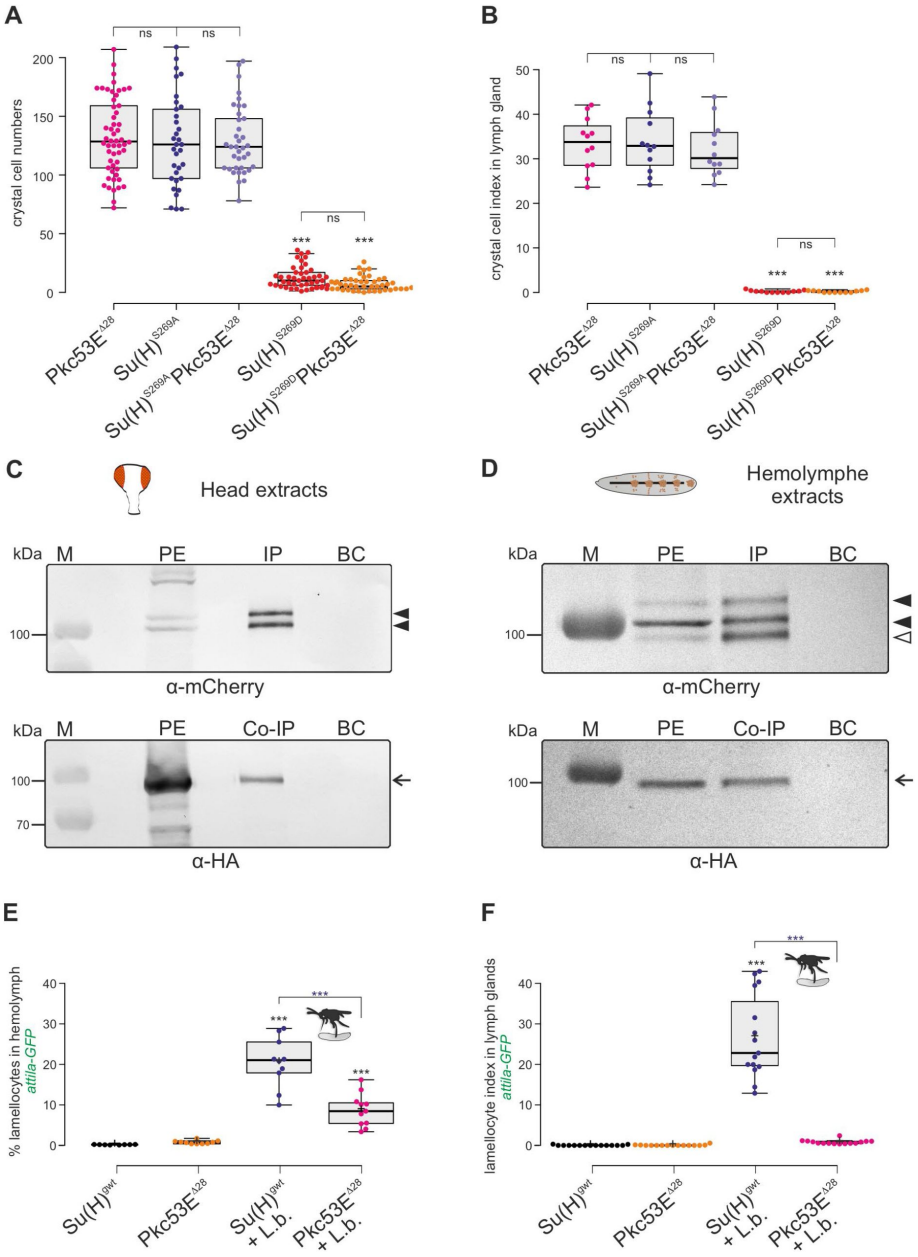

Figure 7.

Pkc53E interacts with Su(H) and is immune compromised

(A) Larval crystal cell number and (B) crystal cell indices in lymph glands were determined in the given genotypes. Each dot represents one analysed larva (n=33-70) in (A) or lymph gland lobus (n=12) in (B). ANOVA for multiple comparisons, Tukey-Kramer's approach relative to controls with *** p≤0.001; p>0.05 ns (not significant).

(C,D) Co-immuno-precipitation of Pkc53E^{HA} with Su(H)$^{gwt\text{-}mCh}$ protein. RFP-Trap IP was performed on protein extracts from 400 heads (C) and 25 third instar larvae (D), respectively. UAS-Pkc53E-HA expression was induced with *Gmr*-Gal4 in the head or *hml*-Gal in the hemolymph. Endogenous mCherry-tagged Su(H) was trapped and detected with α-mCherry antibodies (black arrowheads). The lowest band from the hemolymph is presumably a degradation product (open arrowhead in (D)). HA-tagged Pkc53E was specifically co-precipitated as detected with anti-HA antibodies (arrow). 10% of the protein extract (PE) used for the IP-Trap was loaded for comparison. BC describes the Trap with only agarose beads as a control. M, pre-stained protein ladder; protein size is given in kDa.

(E,F) Quantification of lamellocytes labelled with the *atilla*-GFP reporter in the circulating hemolymph (E) or in the lymph glands (F), in un-infested conditions or upon wasp infestation as indicated. **(E)** Fraction of GFP-positive lamellocytes relative to the total of DAPI-stained hemocytes in the pooled hemolymph from ten larvae. Each dot represents one larval pool (n=12 experiments). **(F)** Lamellocyte index, i.e. number of GFP-labelled cells relative to the size of the lymph gland (n=15). Statistical analysis by ANOVA multiple comparisons with Tukey-Kramer's test; *** p≤0.001.

cell formation, Notch directs the crystal cell lineage. Accordingly, a downregulation of Notch activity causes a lack of crystal cells, whereas a gain of Notch activity results in increased numbers (Duvic et al., 2002; Lebestky et al., 2003; Terriente-Felix et al., 2013; Gosh et al., 2015; Frankenreiter et al., 2021). In our earlier work, we have shown that the regulation of Notch activity during hemocyte differentiation is independent of the general Notch antagonist Hairless. Instead, it relies on the post-translational modification of the *Drosophila* CSL protein Su(H) (Frankenreiter et al., 2021). Phosphorylation at Ser269 in the beta-trefoil domain of Su(H) impedes DNA-binding activity, and hence the capability of Su(H) to act as a transcriptional regulator in the context of blood cell development, without affecting Su(H) protein expression (Nagel et al., 2017; Frankenreiter et al., 2021). Accordingly, phospho-deficient *Su(H)*^{S269A} mutants develop an excess of crystal cells. Now we provide evidence that Su(H) phosphorylation is likewise involved in parasitoid wasp defense, pointing to a dual use of the Notch pathway in blood cell homeostasis as well as in stress response, which is typical for the myeloid system (Banerjee et al., 2019).

The primary cellular immune response of *Drosophila* to fight parasitoid wasp infestation is an encapsulation of the parasite egg to terminate its further development. Albeit metabolically extremely costly, *Drosophila* larvae have to remodel their entire hematopoietic system to generate the masses of lamellocytes required for the encapsulation (reviewed in Letournau et al., 2016; Kim-Jo et al., 2019; Csordás et al., 2021; Hultmark & Ando, 2022). There are two major sources for the lamellocytes. One is the transdifferentiation of circulating plasmatocytes, released from the sessile compartment and proliferating upon immune challenge (Márkus et al., 2009; Honti et al., 2010; Stofanko et al., 2010; Vanha-Aho et al., 2015; Anderl et al., 2016). Second is a massive expansion of prohemocytes in the lymph gland followed by a differentiation to lamellocytes and their release due to the premature disintegration of the gland (Lanot et al., 2001, Sorrentino et al., 2002, Louradour et al., 2017; Cho et al., 2020). Wasp infestation hence provokes a biased commitment to the lamellocyte lineage at the expense of crystal cells, as the two lineages are mutually exclusive (Krzemien et al., 2010; Tattikota et al., 2020; Cho et al., 2020).

The processes underlying the *Drosophila* immune response to wasp parasitism are well-understood, sharing many molecular details with the inflammatory responses of vertebrates (reviewed in Letourneau et al., 2016; Kim-Jo et al., 2019; Banerjee et al., 2019; Kharrat et al., 2022). Interestingly, sterile injury of *Drosophila* larvae initiates a likewise immune response covering all aspects of wasp-mediated immune challenge (Evans et al., 2022). The epidermal penetration by the wasp ovipositor causes a burst of hydrogen peroxide at the injury site via the activation of the NADPH oxidase DUOX. The oxidative stress then induces a systemic activation of Toll/NF-κB and JNK-signaling within circulating hemocytes as well as within the cells of the posterior signaling center of the lymph gland. Following cytokine release, JAK/STAT and EGFR signalling pathways are activated non-cell autonomously driving the trans/differentiation of pro/hemocytes to lamellocyte fate and lymph gland dispersal (Nappi et al., 1995; Schlenke et al., 2007; Sinenko et al., 2011; Gueguen et al., 2013; Razzell et al., 2013; Louradour et al., 2017; Chakrabarti and Visweswariah, 2020; Evans et al., 2022; reviewed in Letourneau et al., 2016; Banerjee et al., 2019). Whereas the switch to lamellocyte fate is well elaborated, less is known about the processes underlying the simultaneous suppression of crystal cell fate. Obviously, this step requires a downregulation of Notch signalling activity, as the Notch pathway is instrumental to crystal cell fate in all the hematopoietic compartments (Duvic et al., 2002; Lebestky et al., 2003; Schlenke et al., 2007; Small et al., 2014; reviewed in Banerjee et al., 2019). Our work now reveals that a parasitoid wasp attack causes a phosphorylation of Su(H) on Ser269 as means of an efficient, rapid and reversible way to inhibit Notch activity. This idea is consistent with earlier observations, whereby wasp parasitism quenched the expression of a Su(H)-lacZ reporter (Small et al., 2014). Moreover, we provide evidence that Pkc53E is involved in Su(H) phosphorylation during blood cell development as well as during parasitoid wasp-defense in *Drosophila*. The antagonistic role of Pkc53E in blood cell homeostasis is fully consistent

with earlier reports demonstrating that a loss of Pkc53E activity increased the expansion of prohemocytes in a genetic model of myeloid leukemia in *Drosophila* (Sinenko et al., 2010). Moreover, the involvement of PKCs in wasp defense is not completely unexpected given their well-documented role in the immune responses of mammals and *Drosophila* alike. For example, *Drosophila* flies ensure a healthy gut-microbiota homeostasis by modulating DUOX activity as a pathogen-specific defense line (reviewed in Kim and Lee, 2014). DUOX activation and ROS production, induced by gut pathogens was shown to be mediated by Pkc53E and Ca^{2+} via phospholipase Cβ (Ha et al., 2009; Lee et al., 2015). Sterile wounding of the *Drosophila* embryo triggers an instantaneous Ca^{2+} flash followed by hydrogen peroxide production (Razzel et al., 2013). It is conceivable that the epidermal breach by the wasp's sting similarly induces a Ca^{2+} flash that may spark Pkc53E activation as a result. However, PKCs may be activated directly by oxidative modification even in the absence of Ca^{2+}. Moreover, they may promote endogenous ROS production in a positive feed back loop (reviewed in Cosentino-Gomes et al., 2012), that could support the systemic response in the larval lymph gland.

The role of PKCs in the mammalian hematopoietic and immune systems is well documented. PKCs in this context act redundantly, and presumably, this holds also for *Drosophila* where several PKCs exist. Upon activation, PKCs may translocate into the nuclear compartment, where they can directly influence gene expression programs, for example by piloting chromatin factors as substrates, pivotal for regulating immune cell differentiation (reviewed in Lim et al., 2015). PKCs, including PKCα, are present in CD34+ long-term hematopoetic stem cells. Moreover, specific roles in both the myeloid and the multilymphoid lineages are known. Notably, some PKC isoforms including PKCα may play a role in Notch-dependent T-cell development. Notch pathway activity is indispensable for T-cell lineage commitment of early thymic progenitors, however, is rapidly downregulated after the β-selection phase. As treatment with phorbol ester upregulates the transcription of TCRα and β chains, a role of PKC in Notch-dependent commitment of αβ T-cells has been proposed. With the expressing of the invariant TCR components, fate commitment of T-cells driven by Notch signaling is completed. Subsequent cell survival and differentiation into CD4+CD8+ double positive cells, however, depends on the formation of a pre-TCR complex and requires PKCα activity (reviewed in Altman and Kong, 2016; Yui and Rothenberg, 2014). During initial T-cell development, Notch1 signaling increases in intensity in the pre-β-selected thymocytes due to an autoregulatory feedback loop directly controlled by E proteins and by Notch1-CSL itself. Following β-selection, as Notch signaling activity becomes dispensable, Notch1 is abruptly downregulated at the transcriptional level. This rapid downregulation is mediated by the inhibition of the E proteins (Yashiro-Ohtani et al., 2009), however, might in addition involve the phosphorylation of CSL by PKCα. Cell fate in more mature thymocytes is then fixed by the silencing of Notch target genes through chromatin modulators (reviewed in Yui and Rothenberg, 2014).

Appropriate silencing of Notch signaling activity in the course of T-cell development is of utmost importance, as prolonged Notch activity during β-selection predisposes the T-cells to leukemic transformation. Phosphorylation of CSL proteins by PKCα kinase offers a way for a rapid and reversible deactivation of Notch signals not only in *Drosophila* but also in the mammalian system. In fact, the amino acid sequences harboring the respective Serine residue in the CSL beta-trefoil domain are completely conserved between vertebrates and invertebrates (Wilson and Kovall, 2006; Nagel et al., 2017), raising the possibility of a likewise regulatory mechanism in mammalian hematopoiesis and immunity. Indeed, respective phosphorylation of the human CSL protein at the homologous position Ser195 was observed in human embryonic stem cells, where differentiation was induced by phorbol ester treatment (Rigbolt et al., 2011), consistent with an involvement of PKCα in this context as well. In conclusion, our work uncovers an important role for PKC-mediated downregulation of Notch activity via CSL-phosphorylation in blood cell homeostasis and in the immune response to parasitoid wasp infestation in *Drosophila*. Future work may uncover, whether the same mechanisms apply to mammalian hematopoiesis and immunity as well.

Materials and Methods

Key resources are listed in S1 Table

Maintenance of parasitoid wasps and infection assay of Drosophila melanogaster

Leptopilina boulardi (*L. boulardi*), *Leptopilina heterotoma* (*L. heterotoma*) and *Asobara japonica* (*A. japonica*) were kindly provided by B. Häußling and J. Stöckl, Bayreuth, Germany (Weiss et al., 2015). Wasp species were co-cultured with wild-type *Drosophila* larvae at room temperature. To this end, about forty 3-5 days old female wasps and twenty male wasps were co-incubated with second instar larvae for 5-7 days at room temperature. Every other day, fresh drops of honey water were added to the vial plug for feeding the wasps. After two weeks, all hatched flies were discarded. Wasps emerge about 30 days after the infestation.

For the infection assays, 50-100 staged *Drosophila* late second/early third instar larvae of the respective genotype were transferred onto apple juice plates with fresh yeast paste. 30 females and 20 males of the wasps aged between 3-6 days were added to the larvae, allowing to infect them for 4-6 hours. Afterwards, wasps were removed and infected larvae were allowed to develop further in vials with normal fly food. Wasps were only used once for each infection. After infestation, third instar larvae were prepared, or the survival rate of wasps versus *Drosophila* imago was recorded.

Fly work and genetic analyses

Fly crosses were performed with 30-40 virgin females and 20 males to avoid overcrowding and stress. Combination/recombination of fly stocks was monitored by PCR-genotyping using primers listed in S2 Table. A complete list of the Kinase mutant flies tested in the 'larval kinase screen' is found in **Fig.3** – supplement Table 3. As reporter lines served *atilla*-GFP (BL23540) and *PPO3*-Gal4 UAS mCD8-GFP (named *PPO3::GFP*, Dudzic et al., 2015). For Gal4/UAS based overexpression and RNAi-mediated knockdown, we used *hml*-Gal4 (BL30141), *lz*-Gal4 (Lebestky *et al*., 2000; obtained form M. Crozatier, Université de Toulouse, France), UAS-*μMCas9* (VDRC 340002), UAS-HA-Pkc53E (this study), UAS-*white-RNAi* (BL31231) and UAS-*sgRNA-Pkc53E* (VDRC341127). The strain *vasa-φC31*, *96E-attB*/TM3 (Bischof *et al*., 2007) served for the generation of UAS-HA-Pkc53E flies. Su(H) controls and mutants comprised: $Su(H)^{gwt}$, $Su(H)^{gwt\text{-}mCh}$, $Su(H)^{S269A}$ and $Su(H)^{S269D}$/*CyO-GFP*, (Praxenthaler *et al*., 2017; Frankenreiter et al., 2021). $Su(H)^{S269A\text{-}mCh}$ flies were produced in this study by a C-terminal in frame fusion of mCherry to the $Su(H)^{S269A}$ mutant gene followed by genomic integration of the construct via gene engineering as outlined before (Praxenthaler *et al*., 2017).

Generation of the UAS-HA-Pkc53E fly line

Pkc53E cDNA (DGRC GH03188) was PCR amplified and subcloned via *Xho*I/*Xba*I in a modified pBT-HA vector, harbouring three copies of an HA-Tag generated via annealed oligos cloned into *Acc*65I/*Xho*I to generate pBT-3xHA-*Pkc53E*. HA-*Pkc53E* was then shuttled via *Acc*65I/*Xba*I in likewise opened pUAST-attB vector (Bischof et al., 2007). All cloning steps were sequence verified. Primers used for cloning are included in S2 Table. Transgenic fly lines were then generated with the help of the PhiC31 integrase-based system using 96E as landing site (Bischof et al., 2007).

RT-PCR of Pkc53E$^{\Delta 28}$ null mutants

Poly(A)$^{+}$ RNA was isolated from 50 third instar larvae (*Pkc53E*$^{\Delta 28}$ and $y^{1}w^{67c23}$) using PolyATract System Kit 1000 (Promega, Mannheim, Germany) according to the manufacturer's protocol, followed by a DNase I treatment (New England Biolabs GmbH, Frankfurt, Germany, #M0303). Subsequent cDNA synthesis was conducted with qScriber cDNA Synthesis Kit (highQu, Kraichtal, Germany) according to the supplier's protocol. For amplification, a *Pkc53E* primer pair overlapping the last three introns was chosen (Pkc53E_RT-PCR UP and Pkc53E_RT-PCR LP). Tubulin 56D primers (Tub56D_229 UP and Tub56D_507 LP) were used as internal controls. For primers, see S2 Table.

Analyses of *Drosophila* hematopoetic cells and tissues

Determination of sessile larval crystal cells

Larval crystal cells were counted according to Frankenreiter *et al*., 2021. Briefly, staged wandering third instar larvae of the respective genotype were heated to 60°C for 10-12 min. Pictures of the posterior dorsal side were taken with a Pixera camera (ES120, Optronics, Goleta, USA) mounted to a stereo-microscope (Wild M3Z, Leica, Wetzlar, Germany) with Pixera Viewfinder 2.5. Melanized crystal cells appear as black dots, and were counted in the last two larval segments with *ImageJ* 1.51 software using *Cell Counter* tool. 25-74 larvae were scored for the statistical evaluation. For the PMA-feeding experiment, 20-30 developmentally synchronized second instar larvae were selected and grown for 24 h in complete dark at 25°C on fly food with 500 µl of 1 mM Phorbol-12-myristat-13-acetat (PMA, Sigma-Aldrich, St. Louis, USA) added to the surface. Subsequently, wandering third instar larvae were heated and analysed as above.

Visualization and quantification of lamellocytes

The lamellocyte specific reporters *atilla*-GFP or *PPO3*::GFP strains were re/combined with *Su(H)*^{gwt}, *Su(H)*^{S269A} or *Pkc53E*$^{\Delta 28}$ alleles by genetic means. Late second/early third larval instars were infested by *L. boulardi* and the number of lamellocytes was determined two days later and compared with those observed in non-infested larvae. Larvae were washed thoroughly in cold PBS and dried with a tissue and teared apart. The hemolymph of 10 larvae each was collected with a 20 µl Microloader tip (Eppendorf, Hamburg, Germany) and placed on a slide with 7 µl of Vectashield mounting medium containing DAPI (BIOZOL, Eching, Germany). GFP-positive cells, i.e. lamellocytes were counted in relation to the total number of DAPI labelled hemocytes with a Zeiss Axioskop and a PlanNeofluar 20x objective. 8-10 independent bleedings were performed each.

Immunostaining and documentation of larval lymph glands

Larval lymph glands were prepared one-two days after infection and treated as described before (Frankenreiter *et al*., 2021). For comparison, non-infested lymph glands were prepared. Primary antibodies used for staining: mouse anti-Hnt for crystal cells (DSHB 1G9, RRID: AB_528278, 1:20) and guinea pig anti-Pzg as nuclear marker (Kugler and Nagel, 2007; 1:500). GFP signals were monitored directly. Secondary fluorescent antibodies were from Jackson Immuno-Research Laboratories (obtained from Dianova, Hamburg Germany, 1:250 each). Mounted tissue was documented with a Zeiss Axioskop coupled with a BioRad MRC1024 confocal microscope using LaserSharp software 2000. For statistical evaluation at least 12 primary lobes were documented and statistically analyzed by using *Image J* software (Schindelin et al., 2012). Indices represent the number of cells in relation to the size/area of the tissue (in pixel) x 10000.

Determination of kinases and kinase assays

Screening of protein kinase candidates in silico and in vitro

To search for potential candidates in silico, GPS3.0 software was used at the lowest threshold levels, including the 40 kinases with the highest difference between score and cut-off value (Xue *et al*., 2011). The corresponding *Drosophila* kinases were determined with the help of flybase according to (Morrison et al., 2000). For the *in vitro* screen, a 0.5kb cDNA fragment (741-1242) encoding the Su(H) beta-trefoil domain (codons 247-414), was PCR-amplified and cloned via *Bam*HI/ *Eco*RI into pGEX-2T vector (Smith and Johnson, 1988) for bacterial expression and purification of the BTD-GST fusion protein. Primers used for cloning are included in S2 Table. ProQinase GmbH (Freiburg, Germany) provided the 'KinaseFinder assay service'. Briefly, BTD-GST and ^{33}P-ATP served as substrates for 245 human Ser/Thr kinases in multi-well plates, analysed in a microplate scintillation reader. (A) Activity of each kinase was determined, (B) corrected for substrate background activity, and (C) auto-phosphorylation (kinase activity without substrate). A ratio value between phosphorylation of BTD-Su(H) and kinase auto-phosphorylation >1 (A-B/C) was considered as significant.

In vitro ADP-Glo^{TM} kinase assay

Drosophila pBT-3xHA-*Pkc53E* was mutated to generate the pseudo-activated form *Pkc53E*^{EDDD} (A34E/T508D/T650D/S669D) stepwise by site directed mutagenesis using the Q5® Site directed Mutagenesis Kit (New England Biolabs, Frankfurt, Germany). Primers used for mutagenesis are included in S2 Table. *Pkc53E* as well as *PKC53E*^{EDDD} were then shuttled into a modified pMAL vector (Riggs, 1994) where additional restriction sites for *Acc*65I, *Sac*II and *Xho*I had been included in the multiple cloning site via primer annealing. The MBP-Pkc53E and MBP-Pkc53E^{EDDD} fusion proteins were bacterially expressed and purified with Amylose resin (New England Biolabs, Frankfurt, Germany). Additionally, activated human kinase PKCα was obtained for a positive control (ProQinase, Freiburg, Germany). The PKCα pseudo-substrate PS (RFARLG**S**LRQKNV) (Kochs et al., 1993), the wild type Su(H) peptide S^{wt} (ALFNRLR**S**QTVSTRY) and the phospho-deficient peptide S^{SA} (ALFNRLR**A**QTVSTRY) were obtained (peptides & elephants, Henningsdorf, Germany).

To test kinase activity, the ADP-Glo^{TM} Kinase Assay system (Promega, Madison, USA) was used. Kinase assay reactions were performed in 96 well plates in a volume of 25 µl in the dark. Each reaction contained 100 µM of a kinase substrate peptide, 150 ng purified kinase and 500 µM ultra-pure ATP. 150 nM Phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, USA) was added to some reactions. The mixture was filled up with Kinase reaction buffer (40 mM Tris-HCl, 20 mM $MgCl_2$, 0.1 mg/ml BSA, pH 7.4) and incubated at room temperature for 1 h in the dark. 25 µl ADP-Glo Reagent were added and incubated for 40 min to remove residual ATP. 50 µl of Kinase Detection Reagent was applied to convert ADP to ATP. The luminescent signal was measured after 45 min using GloMax® Discover Microplate Reader (Promega, Madison, USA), kindly provided by the Department of Zoology (190z), University of Hohenheim.

NanoLC-ESI-MS/MS analysis of Su(H) peptides

Nano-LC-ESI-MS/MS experiments were performed by the Mass Spectrometry Unit at the Core Facility Hohenheim (640) on an Ultimate 2000 RSLCnano system coupled to a Nanospray Flex Ion Source and a Q-Exactive HF-X mass spectrometer (Thermo Fisher Scientific, Waltham, USA). Peptides were separated with LTQ-Orbitrap XL coupled to a nano-HPLC operated under the control of XCalibur 4.1.31.9 software (Thermo Fisher Scientific, Waltham, USA). For all measurements using the Orbitrap detector, internal calibration was as described before (Olsen et al., 2005). MS/MS spectra were analyzed using Proteome Discoverer 2.2 (ThermoFisher Scientific, Waltham, United States), verified by manual inspection of the MS/MS spectra (Voolstra et al., 2010).

Generation of an α-pS269 antiserum

Rabbit polyclonal p-S269 Su(H) antiserum was generated by DAVIDS Biotechnology GmbH (Regensburg, Germany) using the synthetic phospho-peptide NLRL**pS**QTVSTRYLHVE. Phospho-specific antibodies were enriched in a depletion-step by affinity purification against the non-phosphorylated peptide.

Immunoprecipitation of mCherry and Myc-tagged Su(H)

400 adult heads or 25 larvae of each genotype were homogenized on ice in 220 µl buffer 1 [150 mM NaCl, 1% Triton X-100, 50 mM Tris-HCl pH 7.5, 0.1% SDS, supplemented with protease inhibitor cocktail (Roche, Basel, Switzerland)] and incubated for 15 min. After a short spin, 20 µl of the supernatant was set aside as input fraction ('protein extract'). The residual supernatant was diluted with 300 µl wash buffer I (see buffer I without Triton X-100). 15 µl of equilibrated magnetic RFP-Trap Agarose beads (ChromoTek, Planegg, Germany) were added, incubated for 1 h at 8°C and washed three times with wash buffer I. mCherry-trapped proteins were resolved on SDS-PAGE; Western blots were probed with rabbit anti-mCherry (GeneTex, Irvine, USA, 1:1000, #GTX128508) and rat anti-HA (Roche, Basel, Switzerland, 1:500, #11867423001). Goat secondary antibodies coupled with alkaline phosphatase (Jackson Immuno Research Laboratories, 1:1000) were used for detection.

HeLa cell culture experiments and Luciferase assays

Generation of HSV-TK 2xMyc-Su(H)-VP16

Two myc-tags were added to *Su(H)* cDNA in pBT (Maier et al., 2011) by insertion of the two annealed oligonucleotides Myc-Tag UP and Myc-tag LP into the *Eco*RI site (for primers, see S2 Table). The construct was subsequently shuttled via *Eco*RI/*Xho*I into pCDNA3.1 (Invitrogen, Thermo Fisher Scientific, Waltham, USA). A VP16 activator domain was then cloned in frame at the C-terminus of 2xMyc-*Su(H)* by replacing the 829 bp *BspE*I/*Apa*I fragment of pCDNA3 *2xMyc-Su(H)* with a respective1060 bp fragment of the pUAST *Su(H)-VP16* construct (Cooper et al., 2000). Then the CMV Promotor of pCDNA3 2xMyc-Su(H)-VP16 was replaced by the HSV-TK Promotor of the pRL TK Vector (Promega, Madison, USA). To this end, the 1023 bp *Bgl*II/*Nhe*I fragment from pRL TK was cloned into the *Bgl*II/*Spe*I opened pCDNA3 *2xMyc-Su(H)*^{VP16} construct.

Transfection of HeLa cells and reporter assay

RBPj^{KO}HeLa cells were cultivated and transfected as described (Wolf et al., 2019). The following constructs were used: pGL3 NRE-reporter (Bray et al., 2005), pCDNA3 HSV-TK 2xMyc Su(H)^{VP16} and pRL TK (Promega, Madison, USA). For the Luciferase assay, 1 x 10^5 HeLa *RBPj*^{KO} cells were seeded in each well of a 12-well cell culture plate. After 24 h the cells were transfected with 500 ng pGL3 NRE-reporter, 460 ng pCDNA3 HSV-TK 2xMyc Su(H)^{VP16} and 40 ng pRL-TK. 4 h after the transfection the cells were treated with 162 nM PMA (Sigma-Aldrich, St. Louis, USA), 162 nM PMA plus 21,4 nM stauporine (STAU, Sigma-Aldrich, St. Louis, USA) or 21,4 nM STAU alone. Control cells were treated with the same volume of DMSO present in the other treatments. 14 hours later, cells were washed twice in PBS pH 7.4 and lysed in 75 µl 1x Passive Lysis Buffer (Promega, Madison, USA). The Dual-Luciferase® Reporter Assay (Promega, Madison, USA) was performed according to the manufacturer's instructions.

Statistical analysis and documentation of data

Statistical significance of collected data was determined by a two-tailed analysis of variance (ANOVA) approach for multiple comparisons according to Dunnett's Test and Tukey-Kramer's Honestly Significance Difference with p-values ***, p≤0.001; **, p≤0.01; *, p≤0.05; not significant,

p>0.5. Boxplots were created with BoxPlotR software (Spitzer et al. 2014). Pictures were assembled using ImageJ, PhotoPaint, CorelDraw and BoxPlotR software.

Data availability

All data are contained within the manuscript.

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Supplemental Materials

Figures Supplement

Figure 2 - figure supplement 1

The α-pS269 antiserum detects the phospho-mimetic Su(H) variant in vitro

Figure 3D – figure supplement 1

MS/MS spectra of the phosphorylated Su(H) peptide

Figure 4 – figure supplement 1

Conservation of Pkc53E and generation of an activated Pkc53E^{EDDD} isoform

Figure 6 – figure supplement 1

The Pkc53E$^{\Delta 28}$ allele is a null mutant

Tables Supplement

Figure 3 – supplement Table 1

List of kinases predicted to recognize S269 in Su(H) as substrate *in silico*

Figure 3 – supplement Table 2

List of kinases accepting the BTD domain of Su(H) as substrate *in vitro*

Figure 3 – supplement Table 3

Fly strains used for the larval crystal cell screen

Figure 3 – supplement Table 4

Larval crystal cell screen

Supplemental Tables, Materials and Methods

S1 Table Key resources

S2 Table Oligonucleotides

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Reviewer #1 (Public Review):

The authors previously showed in cell culture that Su(H), the transcription factor mediating Notch pathway activity, was phosphorylated on S269 and they found that a phospho-deficient Su(H) allele behaves as a moderate gain of Notch activity in flies, notably during blood cell development. Since a downregulation of Notch signaling was proposed to be important for the production of a specialized blood cell types (lamellocytes) in response to wasp parasitism, the authors hypothesized that Su(H) phosphorylation might be involved in this cellular immune response.

Consistent with their hypothesis, the authors show that Su(H)S269A knock-in flies display a reduced response to wasp parasitism and that Su(H) is phosphorylated upon infestation. Using in vitro kinase assays and a genetic screen, they identify the PKCa family member Pkc53E as the putative kinase involved in Su(H) phosphorylation and they show that Pkc53E can bind Su(H). They further show that Pkc53E deficit or its knock-down in larval blood cells results in similar blood cell phenotypes as Su(H)S269A, including a reduced response to wasp parasitism, and their epistatic analyses indicate that Pkc53E acts upstream of Su(H).

Strengths
The manuscript is well presented and the experiments are sound, with a good combination of genetic and biochemical approaches and several clear phenotypes which back the main conclusions. Notably Su(H)S269A mutation or Pkc53E deficiency strongly reduces lamellocyte production and the epistatic data are convincing.

Weaknesses
The phenotypic analysis of larval blood cells remains rather superficial. Looking at melanized cells is a crude surrogate to quantify crystal cell numbers as it is biased toward sessile cells (with specific location) and does not bring information concerning the percentage of blood cells differentiated along this lineage.

In Su(H)S269A knock-in or Pkc53E zygotic mutants, the increase in crystal cells in uninfected conditions and the decreased capacity to induce lamellocytes following infection could have many origins which are not investigated. For instance, premature blood cell differentiation could promote crystal cell differentiation and reduce the pool of lamellocytes progenitors. These mutations could also affect the development and function of the posterior signaling center in the lymph gland, which plays a key role in lamellocyte induction. Similarly, the mild decrease on resistance to wasp infestation (Fig. 2A) could reflect a constitutive reduction in blood cell numbers in Su(H)S269A larvae rather than a defective down-regulation of Notch activity.
Whereas the authors also present targeted-knock down/inhibition of Pkc53E suggesting that this enzyme is required in blood cells to control crystal cell fate (Fig. 6), it is somehow misleading to use lz-GAL4 as a driver in the lymph gland and hml-GAL4 in circulating hemocytes as these two drivers do not target the same blood cell populations/steps in the crystal cell development process.

In addition, the authors do not present evidence that Pkc55E function (and Su(H) phosphorylation) is required specifically in blood cells to promote lamellocyte production in response to infestation.

Finally, the conclusion that Pkc53E is (directly) responsible for Su(H) phosophorylation needs to be strengthened. Most importantly, the authors do not demonstrate that Pkc53E is required for Su(H) phosphorylation in vivo (i.e. that Su(H) is not phosphorylated in the absence of Pkc53E following infestation). In addition, the in vitro kinase assays with bacterially purified Pkc53E (in the presence of PMA or using an activated variant of Pkc53E) only reveal a weak

activity on a Su(H) peptide encompassing S269 (Fig. 4). Moreover, while the authors show a coIP between an overexpressed Pkc53E and endogenous Su(H) (Fig. 7) (in the absence of infestation), it has recently been reported that Pkc53E is a cytoplasmic protein in the eye (Shieh et al. 2023), calling for a direct assessment of Pkc53E expression and localization in larval blood cells under normal conditions and upon infestation. Furthermore, the effect of the PKCa agonist PMA on Su(H)-induced reporter gene expression in cell culture and crystal cell number in vivo is somehow consistent with the authors hypothesis, but some controls are missing (notably western blots to show that PMA/Staurosporine treatment does not affect Su(H)-VP16 level) and it is unclear why STAU treatment alone promotes Su(H)-VP16 activity (in their previous reports, the authors found no difference between Su(H)S269A-VP16 and Su(H)-VP16) or why PMA treatment still has a strong impact on crystal cell number in Su(H)S269A larvae.

Reviewer #2 (Public Review):

Summary: The current draft by Deischel et.al., entitled "Inhibition of Notch activity by phosphorylation of CSL in response to parasitization in Drosophila" decribes the role of Pkc53E in the phosphorylation of Su(H) to downregulate its transcriptional activity to mount a successful immune response upon parasitic wasp-infection. Overall, I find the study interesting and relevant especially the identification of Pkc53E in phosphorylation of Su(H) is very nice. However, I have a number of concerns with the manuscript which are central to the idea that link the phosphorylation of Su(H) via Pkc53E to implying its modulation of Notch activity. I enlist them one by one subsequently.

Strengths: I find the study interesting and relevant especially because of the following:
1. The identification of Pkc53E in phosphorylation of Su(H) is very interesting.
2. The role of this interaction in modulating Notch signaling and thereafter its requirement in mounting a strong immune response to wasp infection is also another strong highlight of this study.

Weaknesses:1. Epistatic interaction with Notch is needed: In the entire draft, the authors claim Pkc53E role in the phosphorylation of Su(H) is down-stream of notch activity. Given the paper title also invokes Notch, I would suggest authors show this in a direct epistatic interaction using a Notch condition. If loss of Notch function makes many more lamellocytes and GOF makes less, then would modulating Pkc53E (and SuH)) in this manifest any change? In homeostasis as well, given gain of Notch function leads to increased crystal cells the same genetic combinations in homeostasis will be nice to see.
While I understand that Su(H) functions downstream of Notch, but it is now increasingly evident that Su(H) also functions independent of Notch. An epistatic relationship between Notch and Pkc will clarify if this phosphorylation event of Su(H) via Pkc is part of the canonical interaction being proposed in the manuscript and not a non-canoncial/Notch pathway independent role of Su(H).

This is important, as I worry that in the current state, while the data are all discussed inlight of Notch activity, any direct data to show this affirmatively is missing. In our hands we do find Notch independent Su(H) function in immune cells, hence this is a suggestion that stems from our own personal experience.

2. Temporal regulation of Notch activity in response to wasp-infection and its overlapping dynamics of Su(H) phosphorylation via Pkc is needed: First, I suggest the authors to show how Notch activity post infection in a time course dependent manner is altered. A RT-PCR profile of Notch target genes in hemocytes from infected animals at 6, 12, 24, 48 HPI, to gauge an understanding of dynamics in Notch activity will set the tone for when and how it is being modulated. In parallel, this response in phospho mutant of Su(H) will be good to see and will support the requirement for phosphorylation of Su(H) to manifest a strong immune response. Second, is the dynamics of phosphorylation in a time course experiment is missing. While the

increased phosphorylation of Su(H) in response to wasp-infestation shown in Fig.2B is using whole animal, this implies a global down-regulation of Su(H)/Notch activity. The authors need to show this response specifically in immune cells. The reader is left to the assumption that this is also true in immune cells. Given the authors have a good antibody, characterizing this same in circulating immune cells in response to infection will be needed. A time course of the phosphorylation state at 6, 12, 24, 48 HPI, to guage an understanding of this dynamics is needed. The authors suggest, this mechanism may be a quick way to down-regulate Notch, hence a side by side comparison of the dynamics of Notch down-regulation (such as by doing RT-PCR of Notch target genes following different time point post infection) alongside the levels of pS269 will strengthen the central point being proposed. Last, in Fig7. the authors show Co-immuno-precipitation of Pkc53EHA with Su(H)gwt-mCh 994 protein from Hml-gal4 hemocytes. I understand this is in homeostasis but since this interaction is proposed to be sensitive to infection, then a Co-IP of the two in immune cells, upon infection should be incorporated to strengthen their point.

3. In Fig 5B, the authors show the change in crystal cell numbers as read out of PMA induced activation of Pkc53E and subsequent inhibition of Su(H) transcriptional activity, I would suggest the authors use more direct measures of this read out. RT-PCR of Su(H) target genes, in circulating immune cells, will strengthen this point. Formation of crystal cells is not just limited to Notch, I am not convinced that this treatment or the conditions have other affect on immune cells, such as any impact on Hif expression may also lead to lowering of CC numbers. Hence, the authors need to strengthen this point by showing that effects are direct to Notch and Su(H) and not non-specific to any other pathway also shown to be important for CC development.

4. In addition to the above mentioned points, the data needs to be strengthened to further support the main conclusions of the manuscript. I would suggest the authors present the infection response with details on the timing of the immune response. Characterization of the immune responses at respective time points (as above or at least 24 and 48 HPI, as norms in the field) will be important. Also, any change in overall cell numbers, other immune cells, plasmatocytes or CC post infection is missing and is needed to present the specificity of the impact. The addition of these will present the data with more rigor in their analysis.

5. Finally, what is the view of the authors on what leads to activation of Pkc53E, any upstream input is not presented. It will be good to see if wasp infection leads to increased Pkc53 kinase activity.

Overall, I think the findings in the current state are interesting and fill an important gap, but the authors will need to strengthen the point with more detailed analysis that includes generating new data and also presenting the current data with more rigor in their approach. The data have to showcase the relationship with Notch pathway modulation upon phosphorylation of CSL in a much more comprehensive way, both in homeostasis and in response to infection which is entirely missing in the current draft.

Reviewer #3 (Public Review):

Diechsel et al. provide important and valuable insights into how Notch signalling is shut down in response to parasitic wasp infestation in order to suppress crystal cell fate and favour lamellocyte production. The study shows that CSL transcription factor Su(H) is phosphorylated at S269A in response to parasitic wasp infestation and this inhibitory phosphorylation is critical for shutting down Notch. The authors go on to perform a screen for kinases responsible for this phosphorylation and have identified Pkc53E as the specific kinase acting on Su(H) at S269A. Using analysis of mutants, RNAi and biochemistry-based approaches the authors convincingly show how Pkc53E-Su(H) interaction is critical for remodelling hematopoiesis upon wasp challenge. The data presented supports the overall

conclusions made by the authors. There are a few points below that need to be addressed by the authors to strengthen the conclusions:

1. The authors should check melanized crystal cells in Su(H)gwt and Su(H)S269A in presence of PMA and Staurosporine?
2. Data for number of dead pupae, flies eclosed, wasps emerged post infestation should be monitored for the following genotypes and should be included: Pkc53EΔ28, Su(H)S269A, Pkc53EΔ28 Su(H)S269A, Su(H)S269D, Su(H)S269D Pkc53EΔ28
3. The exact molecular trigger for activation of Pkc53E upon wasp infestation is not clear.
4. The authors should check if activating ROS alone or induction of Calcium pulses/DUOX activation can mimic this condition and can trigger activation of Pkc53E and thereby cause phosphorylation of Su(H) at S269
5. Does Pkc53E get activated during sterile inflammation?