The Zic family homologue Odd-paired regulates Alk expression in Drosophila

Patricia Mendoza-García¹,², Fredrik Hugosson¹, Mahsa Fallah², Michael L. Higgins³, Yasuno Iwasaki³, Kathrin Pfeifer¹, Georg Wolfstetter¹, Gaurav Varshney², Dmitry Popichenko², J. Peter Gergen³, Korneel Hens⁴, Bart Deplancke⁵, Ruth H. Palmer¹*

¹ Department of Medical Biochemistry and Cell Biology, Institute of Biomedicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden, ² Department of Molecular Biology, Umeå University, Umeå, Sweden, ³ Department of Biochemistry and Cell Biology, Stony Brook University, Stony Brook, New York, United States of America, ⁴ Centre for Neural Circuits and Behaviour, University of Oxford, Oxford, United Kingdom, ⁵ Laboratory of Systems Biology and Genetics, Lausanne, Switzerland

* ruth.palmer@gu.se

Abstract

The Anaplastic Lymphoma Kinase (Alk) receptor tyrosine kinase (RTK) plays a critical role in the specification of founder cells (FCs) in the Drosophila visceral mesoderm (VM) during embryogenesis. Reporter gene and CRISPR/Cas9 deletion analysis reveals enhancer regions in and upstream of the Alk locus that influence tissue-specific expression in the amnioserosa (AS), the VM and the epidermis. By performing high throughput yeast one-hybrid screens (Y1H) with a library of Drosophila transcription factors (TFs) we identify Odd-paired (Opa), the Drosophila homologue of the vertebrate Zic family of TFs, as a novel regulator of embryonic Alk expression. Further characterization identifies evolutionarily conserved Opa-binding cis-regulatory motifs in one of the Alk associated enhancer elements. Employing Alk reporter lines as well as CRISPR/Cas9-mediated removal of regulatory elements in the Alk locus, we show modulation of Alk expression by Opa in the embryonic AS, epidermis and VM. In addition, we identify enhancer elements that integrate input from additional TFs, such as Binou (Bin) and Bagpipe (Bap), to regulate VM expression of Alk in a combinatorial manner. Taken together, our data show that the Opa zinc finger TF is a novel regulator of embryonic Alk expression.

Author summary

The Alk receptor tyrosine kinase is employed repeatedly during Drosophila development to drive signaling events in a variety of tissues. The spatial and temporal expression pattern of the Alk gene is tightly regulated. Identifying factors that influence the expression of Alk is important to better understand how Alk signaling is controlled. In this paper we characterize cis-regulatory sequences in the Alk locus and the transcription factors that bind them to govern Alk expression in the Drosophila embryo. Using a robotic protein-DNA interaction assay, we identified the Zic family transcription factor Odd-paired as a factor that binds to regulatory elements in the Alk locus. Binding of Odd-paired to Alk cis-regulatory elements varies spatially, revealing a requirement for
additional transcription factors such as the NK3 and FoxF orthologues Bagpipe and Biniou in a subset of Alk-expressing tissues. Our findings provide new insight into the dynamics underlying temporal and spatial regulation of the Alk receptor during embryogenesis.

Introduction

During embryogenesis, the Anaplastic Lymphoma Kinase (Alk) receptor tyrosine kinase (RTK) is dynamically expressed predominantly in the primordia of the visceral mesoderm (VM), the developing CNS, the amnioserosa (AS) and in a restricted manner in the epidermis [1]. Alk plays a critical role during VM development, where it is activated in response to the secreted ligand Jelly Belly (Jeb) driving the Ras/MAPK/ERK pathway [2–5]. This leads to expression of founder cell (FC) specific transcription factors (TFs) such as Hand [6], optomotor-blind related-1 (org-1) [4] and factors important in the muscle cell fusion process like dumbfounded/kin of irre (duf/kirre) [3–5]. Jeb/Alk signaling also leads to downregulation of fusion competent myoblast (FCM)-specific factors such as sticks and stones (sns) [7] and Verprolin 1 (vrp1) [8–10]. In addition, Alk signaling in the VM modulates the subcellular localization of the Gli-family TF Lame duck (Lmd), resulting in Lmd translocation from the nucleus to the cytoplasm [11]. Thus, signaling regulated by Jeb/Alk is critical for embryonic FC-specification and the subsequent fusion with FCMs to form a functional larval midgut muscle [2–5].

While we and others have previously identified and characterized several important components and targets of the Alk RTK signaling pathway, little is currently understood about the molecular mechanisms regulating the spatial and temporal expression of the Alk receptor itself. Development of the early VM requires the activity of the NK4/msh-2-like homeobox TF Tinman (Tin) for dorsal mesoderm differentiation, as well as the NK3 and FoxF orthologues Bagpipe (Bap) and Biniou (Bin) [12–15]. Interestingly, the expression patterns of bap and bin in the VM primordia are similar to that of Alk [15]. In addition, ChIP-on-chip studies have shown the region upstream of Alk gene to be occupied by several mesodermally expressed TFs, such as Bin, Bap, Twist (Twi), Tin and Myocyte enhancer factor 2 (Mef2) at different time points during embryogenesis [16, 17]. While binding of these factors has been documented, their importance in the regulation of Alk transcription in the VM has only been initially characterized in case of Tin [16, 17].

Here we address regulation of Alk expression during embryogenesis. We have employed a combination of in vitro and in vivo approaches to identify and characterize Alk-specific enhancer elements, including high throughput yeast one-hybrid screening (Y1H) with a library of Drosophila TFs [18]. This Y1H screen identified the zinc finger TF Odd-paired (Opa) as binding to an evolutionary conserved cis-regulatory module (CRM) within one of the Alk-associated enhancer regions. In agreement with these findings, opa mutants displayed a complete loss of Alk expression in the epidermis and reduced levels of Alk in the VM. Furthermore, CRISPR/Cas9-mediated deletion of the Opa binding site containing region in the Alk locus resulted in a reduction of VM Alk protein together with loss of Alk expression in both the AS and embryonic epidermis, indicating that Opa plays an important role in tissue-specific Alk expression during embryogenesis. We have also identified additional enhancer regions regulated by the Bin and Bap TFs, likely together with additional TFs, that work with the Opa binding CRM to regulate Alk expression in the VM in a combinatorial manner.
Results
Identification of regulatory regions involved in Alk expression during embryogenesis

To study Alk expression during embryogenesis, we employed transgenic GAL4-lines containing overlapping DNA sequences corresponding to Alk 5-prime upstream regions (Fig 1A, S1 Fig), aiming to identify regulatory elements with activity in the visceral mesoderm (VM). AlkEI6.5-GAL4 was previously described [1] as driving expression in the trunk VM with stronger expression in founder cells (FCs) (Fig 1B, stage 11, arrowhead). We also noted that the AlkEI6.5-GAL4 driver was expressed in the amnioserosa (AS), in keeping with earlier observations that Alk mRNA is expressed in the dorsal-most region of the embryo corresponding to the presumptive AS at the early gastrulation stage (S2A and S2B Fig) [1]. We next analyzed AlkE4-GAL4, which contains 2.4 kb of the AlkEI6.5-GAL4 region and an additional 1.6 kb upstream (4.0 kb in total). This GAL4-driver promotes expression in a similar pattern to AlkEI6.5-GAL4, suggesting this DNA region also contains regulatory elements involved in Alk transcriptional regulation (Fig 1C). In addition, AlkE2.7-GAL4, covering a shorter sequence within AlkEI6.5 and AlkE4, displays activity in the entire trunk VM, being considerably stronger in FCs (Fig 1D, arrowhead).

To ensure the specificity of our transgenic lines for the Alk locus flanking genes we performed in situ hybridization on both neighboring genes namely CG5065 (upstream) and gprs (downstream) (Fig 1A, S3 Fig). Neither CG5065 nor gprs is expressed in a pattern similar to that of Alk in the VM, suggesting that any VM expressing region identified flanking the Alk locus may be involved in the regulation of Alk transcription.

The elevated level of expression of AlkEI6.5-GAL4 and AlkE2.7-GAL4 in FCs compared with other cells of the developing VM suggests Alk may respond to its own signaling. Since signaling in the FCs is driven by activation of Alk by its ligand Jelly Belly (Jeb), we examined expression of AlkE2.7-GAL4 in either the absence of Alk activity (Alk1/Alk10), or upon activation of Alk by overexpression of Jeb in the VM. AlkE2.7-GAL4 expression in the FCs was reduced in Alk1/Alk10 mutants (Fig 1F; arrowhead). In contrast, overexpression of Jeb resulted in robust expression of AlkE2.7-GAL4 in all cells of the VM (Fig 1G; arrowhead). These results suggest that Alk expression in the VM is positively regulated by Alk signaling, representing a positive feedback loop. Thus, we have identified CRMs in the 5’ region of the Alk locus that promote Alk expression in the presumptive amnioserosa and developing VM. Additionally, our preliminary GAL4 analysis suggests the presence of inhibitory modules within this region that likely contribute to the overall regulation of Alk expression.

Analysis of Alk enhancer regions in vivo by CRISPR/Cas9-mediated deletion identifies a critical role for the Alk-RB promoter

ChIP experiments performed by the Furlong laboratory have identified a 547 bp CRM (MesoCRM-880) overlapping the AlkE2.7 fragment that binds Bin, Bap, Mef, Tin and Twi TFs [16] (shown schematically in Fig 1A, S1 Fig). Later analysis by the Frasch group identified a 1,984 bp region (AlkE301) in a genome wide Tin ChIP analysis that drives expression in the VM [17] (shown schematically in Fig 1A, S1 Fig). Together with our GAL4 analyses these results suggest that the Alk-RB promoter may be important for the VM expression of Alk. To functionally address the role of Alk-RB we generated deletion mutants targeting the Alk-RB isoform with CRISPR/Cas9 [19–21], employing two independent single guide RNA (sgRNA) combinations. This resulted in genomic deletions of 1053 bp (represented by AlkAB.RB.122) or 1325 bp (represented by AlkAB.RB.15.16.2) in the region of the Alk-RB 5’UTR (Figs 1A and 2A;
Fig 1. Reporter gene analysis identifies putative regulatory elements responsible for Alk expression. (A) Genomic organization of the Alk locus (green) and the neighboring genes CG5065 and gprs (light gray). Intron-exon structure of both Alk-RA and Alk-RB transcripts (Alk open reading frame in white) and the analyzed reporter constructs (blue lines) are shown below. The MesoCRM-880 and AlkE301 CRMs identified in previous ChIP analyses are depicted as grey lines and the 3.6 kb region subjected to Y1H analysis is shown in red (dashed lines). CRISPR/Cas9 deletions disrupting the Alk-RB promoter, AlkΔRB_1 and AlkΔRB_15, are indicated as black dashed lines. (B) AlkE16.5 drives expression in the trunk VM at stage 11; with strongest expression in the founder cells (FCs) (close up; FCs, arrowhead). (C) AlkE4 shows a slightly more restricted VM expression pattern compared to that of AlkE16.5. (D) Similarly, AlkE2.7 is expressed in the entire VM with marked stronger expression in the
FCs (close up, arrowhead). (E-F) AlkE2.7 expression in the FCs is responsive to Alk signaling. lacZ expression in Alk^1/Alk^15 embryos is weaker when compared to Alk^10 heterozygote balanced controls (arrowhead; compare β-gal heatmaps in E and F; note: epidermal β-gal expression in control (E) is due to presence of lacZ balancer; Alk protein is observed in Alk^1/Alk^15 animals (F) as these alleles encode non-functional Alk protein truncations detected with anti-Alk). (G) Ectopic expression of the Alk ligand Jeb, leads to activation of Alk signaling in all cells of the VM (arrowhead) and is marked by expression of Org-1 in blue resulting in increased lacZ expression from AlkE2.7 in all cells of the VM (compare β-gal heatmaps in E and G). Close up regions in E-G are indicated with dashed boxes. Scale bars: 50 μm and 10 μm (embryo and close up, respectively).

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S1 Fig; S1 Table). Both homozygous mutants were embryonic lethal. We further examined the visceral morphology of homozygous Alk^ARBl.22.2 mutant embryos and control siblings using Fasciclin III (FasIII) as marker for differentiated VM. In control embryos FasIII was expressed in the visceral musculature surrounding the entire midgut, which at later stages of embryogenesis is subdivided into four chambers (Fig 2B; arrowhead). In Alk^ARBl.22.2 embryos, FasIII-
positive midgut muscles were absent while FasIII-expression could still be detected in the embryonic foregut and hindgut respectively (Fig 2C), resembling the Alk mutant phenotype [2]. In agreement with their mutant phenotype, Alk<sup>ARB.1.22.2</sup> mutants lacked detectable Alk mRNA and protein in the VM (Fig 2K, S4 Fig) compared to wild-type animals (Fig 2E and 2H, S4 Fig), while Alk expression levels in the CNS were similar to those observed in control embryos (Fig 2I and 2L; S4 Fig). Alk expression was also lost in the AS and epidermis of Alk<sup>ARB.1.22.2</sup> mutants (Fig 2J and 2K, asterisks). Therefore, expression from the Alk-RB promoter drives Alk expression in the embryonic VM, AS and epidermis and is critical for proper formation of the midgut musculature.

Identification of potential regulators of Alk expression by high throughput yeast one-hybrid screening

A 3.6 kb genomic region that covered the putative VM and epidermal Alk enhancer regions identified in our initial experiments (Fig 1A) was subjected in parallel to high throughput yeast one-hybrid (Y1H) and more detailed reporter gene analyses. Six fragments (denoted AlkEB6 – AlkEB11; S1A Fig; S2 Table) of approximately 700 bp in length, including a ~100 bp overlap between neighboring fragments, were analyzed.

Embryonic lacZ reporter activity was observed with only two of the DNA fragments studied, namely AlkEB8 and AlkEB9 (Fig 3A–3E). AlkEB8 displayed weaker activity in the VM than that observed with AlkEB9 (Fig 3B, 3B”, 3D and 3D”, arrowheads; quantified in S5 Fig). In addition to VM expression, AlkEB9 was also expressed in the AS and epidermis where it overlapped with Alk protein (Fig 3E, asterisks; S2D Fig). No expression in the AS and epidermis was observed in AlkEB8 (Fig 3C, asterisks; S2C Fig). To further confirm that AlkEB9 contains important enhancer elements for Alk, we performed rescue experiments using AlkE9-GAL4 (Fig 3F–3H). Ectopic over-expression of Alk (AlkEB9-GAL4 >> UAS-Alk) in an Alk<sup>1</sup>/Alk<sup>10</sup> mutant background resulted in a rescue of the embryonic gut phenotype (Fig 3H). Therefore, the AlkEB9 genomic region contains sufficient regulatory information to allow rescue of the embryonic Alk VM expression.

High throughput Y1H was carried out on the same six fragments employing a library of Drosophila TFs fused to the yeast GAL4 activation domain [18] (Fig 1A, S1 Fig). Based on our reporter gene analysis we focused on the AlkEB9 DNA bait Y1H data set aiming to functionally characterize novel transcriptional regulators of Alk. A set of TFs was identified to bind to the AlkEB9 DNA bait by Y1H screening (Fig 4A). Among these, Odd-paired (Opa) (Fig 4B), Pointed (Pnt), Side and CG14655 bound to the AlkEB9 DNA bait and promoted growth in selective media in all biological replicates performed. We further investigated a role for TFs binding AlkEB9 in Alk transcriptional regulation in vivo, employing paired (prd)-GAL4, which drives expression in alternating parasegments and offers internal control of Alk expression levels in the epidermis. In this assay both Opa and Pnt were identified as potential regulators of Alk, with Opa inducing and Pnt repressing Alk expression (S6 Fig). Of the TFs tested in this study, Opa was the only one that resulted in an increase in Alk protein. We also overexpressed opa with the engrailed (en)-GAL4 driver which resulted in an increase in AlkEB9-lacZ reporter activity as well as Alk protein levels in the epidermis (Fig 4C–4D'), indicating that Opa is sufficient to promote Alk expression. Therefore we focused on a more detailed investigation of the role of Opa Alk transcriptional regulation.

AlkEB9 contains functional Opa binding sites

Employing the JASPAR online prediction tool [22], we were able to identify a potential Opa binding site (BS) in the AlkEB9 sequence, JASPAR_OpaBS (GACCTCCGGCTG) (Fig 5A and 5B). In
addition, we identified another Opa BS similar to the Opa consensus motif previously reported by [23] and therefore referred to as SELEX_OpaBS (GCGGGGATG) (Fig 5A and 5B). Employing the phastCons database, which identifies evolutionarily conserved elements in a multiple alignment, to analyze this sequence, we found that both binding sites are conserved among Drosophila species (Fig 5B; conservation score in green; Opa BS highlighted in yellow) [24, 25]. We next assessed the ability of Opa to specifically bind these predicted sites by electrophoresis mobility shift assay (EMSA). EMSA was performed on the SELEX_OpaBS and JASPAR_OpaBS sequences, incubating probes with cell lysates from Opa-expressing HEK293 cells in the presence of poly(dI-dC) to prevent non-specific binding. Addition of Opa lysate to the binding reaction resulted in a shift of both SELEX_OpaBS and JASPAR_OpaBS probes and was reversed by addition of 100 fold molar excess of non-labelled probe (Fig 5C and 5D). In contrast, addition of cold probes that were mutated within the SELEX and JASPAR binding sites, based on published data [23], was unable to compete the shift generated upon addition of Opa to the labelled wild-type probe. Furthermore, labelled mutated SELEX_OpaBS and JASPAR_OpaBS probes did not exhibit a mobility shift upon incubation with Opa (Fig 5C and 5D). The above observations led us to characterize...
the interactions of the Opa with the Alk locus by chromatin immunoprecipitation (ChIP). Consistent with Y1H and EMSA analyses, Opa association is detected with a region upstream of the Alk promoter that spans both the SELEX_OpaBS and JASPAR_OpaBS sequences in chromatin from wild-type embryos (Fig 5E).

To address the importance of the JASPAR and SELEX_OpaBS for in vivo Alk transcription we first attempted to identify a minimal region within the AlkEB9 region that could drive VM expression. This analysis led to the identification of a 154 bp fragment including both SELEX and JASPAR Opa binding sites (AlkEB9_OpaBS; schematically shown in S1 Fig) that drives strong VM and epidermal expression, similar to that observed with the 700 bp AlkEB9 fragment (Fig 6A–6F'). Quantification revealed that VM expression from AlkEB9_OpaBS was weaker than that of the 700 bp AlkEB9-lacZ reporter (Fig 6B' and 6E'; S7 Fig), while expression in the epidermis appeared similar in both the 154 bp and 700 bp fragments (Fig 6C' and 6F'; S7 Fig). In order to examine the role of the predicted Opa binding sites, we introduced the same mutations as in our earlier EMSA analysis within the 154 bp AlkEB9_OpaBS minimal region to create AlkEB9_OpaKO-lacZ. Mutation of these binding sites led to a loss of lacZ expression in both the VM and epidermis (Fig 6G–6I', quantified in S7 Fig), implying that the predicted Opa binding sites in AlkEB9 indeed contribute to expression from this element. While mutation of Opa binding sites led to a significant reduction of reporter gene expression in the VM (Fig 6H'; quantified in S7 Fig) this was not complete, in contrast to a complete loss of detectable lacZ activity in the epidermis (Fig 6I'; quantified in S7 Fig). Taken together, these data show that the AlkEB9 genomic region contains sequence-specific binding sites for Opa that regulate expression from Alk enhancer elements.

Opa is required for tissue specific Alk expression during embryogenesis

To further dissect the potential role of Opa as a regulator of Alk expression, we examined opa expression during embryogenesis [26]. opa mRNA can be detected at stage 5 in the ectoderm and mesoderm progenitors spanning the presumptive segmented region of the embryo. At stage 9 opa expression decreases slightly and appears in the neuroectoderm persisting until late
Fig 5. Functional validation of Opa as a putative regulator of Alk. (A) Schematic overview of AlkEB9 element (blue line) and the predicted Opa binding sites, referred to as **SELEX_OpaBS** and **JASPAR_OpaBS** (in red). (B) PhastCons analysis of sequence conservation among 21 Drosophila species (in green) along AlkEB9, including the predicted Opa binding sites (marked with dashed boxes i) and ii). Base resolution of the phastCons analysis for **SELEX_OpaBS** (i) and **JASPAR_OpaBS** sites (ii) is shown, Opa binding sites are highlighted in yellow. (C, D) Binding affinity of Opa to both **SELEX_OpaBS** and **JASPAR_OpaBS** sequences as assessed by EMSA. Opa-induced shifts could be competed by addition of non-labelled probe, but not by unlabeled mutated probes. Sequences of both wild type and mutant probes are indicated, mutated residues are depicted in red. (E) ChIP assay employing either pre-immune serum control (dark grey bars) or anti-Opa serum from the same rabbit (light grey bars) for a control intergenic region (control), an Opa binding region within the slp1 enhancer (DESE-Opa) and a 140 bp region containing both the **SELEX_OpaBS** and **JASPAR_OpaBS** sequences within AlkEB9 (Alk-OpaBS). Enrichment of the different DNA segments in the immunoprecipitates are reported as a percentage of input DNA, with error bars representing the mean ± SD from three technical replicates of the qPCR.

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embryo stages. In the VM, \(opa\) mRNA is observed in a dynamic pattern, where it is expressed in a clustered fashion in PS 3–5 and PS 9–12 (S8 Fig). We next examined the reporter expression of \(AlkEB9\)-\(\beta\)-gal in \(opa\) loss-of-function mutants (\(opa^{1}/opa^{8}\)). While \(AlkEB9\)-\(\beta\)-gal is activated in the entire VM and epidermis in wild-type embryos (Fig 7A and 7A’), \(opa^{1}/opa^{8}\) mutant embryos display only weak reporter activity during embryogenesis (Fig 7B and 7B’; quantified in Fig 7C). The severe developmental defects observed in \(opa^{1}/opa^{8}\) mutants make analysis difficult, however we noted lower levels of Alk protein in the VM and a complete loss of detectable Alk in the epidermis of \(opa\) mutant animals, in agreement with the loss of \(AlkEB9\)-\(\beta\)-gal activity (Fig 7B). These observations were supported by analysis of RNAi-induced Opa knockdown in the developing mesoderm employing \(2xPE-GAL4\) (Fig 7D–7F). We observed that embryos expressing \(opa\) RNAi (\(2xPE-GAL4\)-\(UAS-\(opa\_RNAi\)) displayed a reduction of \(AlkEB9\)-\(\beta\)-gal in the VM at later stages when compared with controls (Fig 7E’, quantified in Fig 7F). Since Opa has been reported to be required for proper midgut formation, with \(opa\) mutants exhibiting an interrupted VM that fails to form midgut constrictions during embryogenesis [26], we also examined Alk signaling in the VM of \(opa\) mutants. \(opa^{1}/opa^{8}\) mutants, examined with the FC-marker Org-1, exhibited Org-1 positive VM FCs, however, the level of Org-1 protein observed was less than in control embryos (S8B and S8C Fig). Since reductions in both Alk and Org-1 protein were seen in \(opa^{1}/opa^{8}\) mutants, we asked whether Opa overexpression was sufficient to drive Alk signaling. As expected, \(bap3-GAL4\)-driven expression of \(Jeb\) in the VM resulted in an increased expression of the \(HandC\_GFP\) FC marker reflecting activation of Alk signaling (S8D–S8D” Fig). In contrast, \(bap3-GAL4\) driven expression of Opa did not
increase HandC-GFP levels (S8E–S8E" Fig). Thus, while Alk signaling may be reduced in opa mutants, Opa is not sufficient to influence FC specification driven by Alk signaling in the embryonic VM.

As a complement to our analysis of opa mutants, we employed the Opa4opt-lacZ transgene as readout for Opa activity, focusing on the epidermis. Opa4opt-lacZ contains four tandem copies of the SELEX determined Opa-BS [23]. In parallel we analyzed the opa32246 lacZ...
enhancer trap which reflects opa expression [26]. We observed expression of both opaSD246 and Opa4opt-lacZ in the embryonic epidermis, coinciding with Alk protein (Fig 7G and 7H), suggesting that Opa is both expressed and active in these cells. Furthermore, a mutant Opa4opt-lacZ transgene, called Opa4opt-KO-lacZ, in which the Opa binding sites are mutated, no longer displayed expression overlapping with Alk in the embryonic epidermis (Fig 7I).

Taken together, this data supports an important role for Opa in driving embryonic Alk transcription, particularly in the epidermis, through the AlkEB9 regulatory region. However, in agreement with our earlier analyses, Alk expression in the VM does not depend only on Opa activity, since Alk protein is still observed in the VM of opa1/opa2 loss of function animals (Fig 7B).

Opa binding sites in the Alk enhancer region regulate Alk protein expression in a tissue-specific context

Given the presence of Opa binding sites proximal to the Alk-RB isoform promoter, together with the loss of reporter gene activity after deletion of these sites, we next addressed their in vivo relevance for Alk transcriptional regulation. CRISPR/Cas9 genome editing was again employed to delete the identified Opa binding sites (Opa-BS) in the AlkEB9 enhancer region of the Alk locus (Fig 8A; S1 Fig). This resulted in isolation of two viable AlkOpaBS mutants: AlkOpaBS_10.28.3 and AlkOpaBS_10.36.1 (Fig 8A and 8E–8I; S1 Fig). Loss of 151 bp containing the Opa binding sites in AlkOpaBS_10.28.3 mutants led to a complete loss of detectable Alk protein in the amnioserosa and epidermis (Fig 8H; S1 Table), indicating this region is essential for Alk expression in these tissues. We also observed reduced Alk protein levels in the VM when compared to control embryos at the same stage (Fig 8H, compare with Fig 8B; quantified in Fig 8N). In close proximity to the Opa binding sites we also observed a cluster of highly scoring JASPAR-predicted binding sites for mesodermal TFs (Bap, Sna and Tfn) in the AlkEB9 genomic region, here designated as meso-BS (Fig 8A; S1 Fig). Deletion of this meso-BS region alone, in AlkOpaBS embryos, does not appear to affect either Alk protein levels or the formation of a fully developed gut (S1 and S9 Figs; S1 Table). Interestingly, AlkOpaBS_10.36.1 removes 178 bp including both the Opa- and the meso-BS sites allowing us to functionally address the contribution of the meso-BS region relative to the Opa binding sites. Deletion of both the meso-BS and the Opa-BS regions (AlkOpaBS_10.36.1) results in viable animals, albeit with reduced Alk protein levels when compared to those in control embryos (Fig 8F, S1 Table). Reduction of Alk protein levels in the VM was noticeably stronger in AlkOpaBS_10.36.1 mutants (Fig 8F and 8H; quantified in Fig 8N). However, the reduced Alk protein levels observed in AlkOpaBS_10.28.3 and AlkOpaBS_10.36.1 were still sufficient to drive Jeb/ Alk signaling in the VM as measured with HandC-GFP reporter expression (Fig 8G and 8I insets), and form a functional gut as visualized by FasIII staining (Fig 8G and 8I).

Since we detected VM expression activity in the overlapping Alk proximal AlkEB8-lacZ reporter (Fig 3B), we explored the contribution of the corresponding region in the Alk locus to regulation of Alk VM expression. To do this we employed CRISPR/Cas9 genomic editing to remove 808 bp covering part of AlkEB9 (312 bp) and the majority of AlkEB8 (647 bp) (represented by AlkEB8) (Fig 8A; S1 Fig, S1 Table). These mutants were homozygous viable, with a wild-type VM morphology (Fig 8K; S1 Fig). Investigation of Alk protein levels in AlkEB8 mutants revealed a decrease, but not complete loss, of Alk in the VM (Fig 8J; quantified in Fig 8N), suggesting that CRM(s) within the AlkEB8 region are not essential but contribute to VM expression of Alk. Expression of Alk in the epidermis was not affected, in agreement with a sole epidermal CRM including the Opa binding sites within the AlkEB9 region. To further exclude the possibility that an essential CRM might be located in the overlap between AlkEB8
Fig 8. The Opa binding site containing CRM is crucial for tissue-specific Alk expression during embryogenesis. (A) Overview of the CRISPR/Cas9 deletions generated (dashed lines) within the AlkEB9 and AlkEB8 enhancer region of the Alk locus (blue lines). Predicted binding sites shown in red.

(B, C) Alk protein is normally expressed in the VM and adjacent epidermis of wild-type embryos at stage 11. Alk activation drives expression of the HandC-GFP reporter in FCs of wild-type embryos (C, inset, stage 11 embryo, green), resulting in midgut formation (C, stage 16, arrowhead).

(D, E) AlkΔRB1.22.2 mutants are indistinguishable from Alk null alleles in the VM, exhibiting loss Alk protein and FC specification (E, inset, stage 11 AlkΔRB1.22.2 mutant embryo) and lack of midgut formation (E, stage 16, arrowhead). (F-I) Both AlkΔCopaBS_10.36.1 and AlkΔCopaBS_10.28.3 mutants lack Alk protein in epidermis and display reduced levels of Alk protein in the VM (F, H, stage 11; quantified in N), although Alk levels in the VM are sufficient to drive FC specification (G, I, insets represent stage 11 AlkΔCopaBS_10.36.1 and AlkΔCopaBS_10.28.3 mutant embryos). (J, K) AlkΔEB8 mutants show reduced Alk protein levels in the VM (J, stage 11; quantified in N), while epidermal expression of Alk appears to be unaffected (J, stage 11). Neither FC specification (K, inset, stage 11 AlkΔEB8 mutant embryo) nor midgut formation (K, stage 16, arrowhead) are impaired in AlkΔEB8 mutants. (L, M) AlkΔCopaBS+EB8 behaves as an Alk null allele in the VM.
lacking detectable Alk protein in the VM and epidermis (L, stage 11), and failing to specify FCs (M, inset, stage 11 Alk^{OpaBS-E88} mutant embryo) or develop a midgut (M, stage 16, arrowhead). (N) Alk^{OpaBS-10.36} A\text{Alk^{OpaBS-10.28.3}} and Alk^{E88} mutants show a significant decrease in Alk protein in the VM when compared to control embryos (n = 10 animals per genotype, * * * p < 0.0001). Scale bars: 50 μm.

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and Alk^{EB8}, we generated a series of overlapping reporter constructs in this area S1 Fig. We did not observe any VM expression activity in this reporter series (S10 Fig), suggesting that two CRMs, one in the region of Alk^{EB8} and one in Alk^{EB9} function together drive VM expression of Alk.

To test the contribution of additional CRMs to VM expression of Alk we extended the Alk^{AEB8} deletion to include the Opa binding sites within Alk^{EB9}. This deletion was denoted Alk^{AOpasBS+E88} (Fig 8A, 8L and 8M; S1 Fig, S1 Table). Alk^{AOpasBS+E88} mutants failed to express Alk protein in the VM, epidermis or AS (Fig 8L), and were homozygous lethal due to lack of FC specification (Fig 8M; inset), supporting our hypothesis of several independent CRMs within this area that are critical for Alk expression in the VM.

Taken together, our analysis identifies a CRM proximal to the Alk-RB isomform promoter that contains Opa binding sites as critical for Alk expression in the embryonic AS and epidermis. This region also contributes to Alk expression in the VM. Further deletion analysis reveals additional CRM(s) located within the Alk^{EB8} fragment that contribute to regulation of Alk VM expression.

Bin and Bap contribute to VM expression of Alk

Previous studies identified CRMs binding Bin, Bap, Twi, and Mef2 in the Alk locus [16, 17]. In particular the ChIP and reporter gene analyses performed by Jin et al. (2013) suggested Tin binding to be important if not essential for Alk expression. We studied expression of Alk protein and the Alk^{EB9-lacZ} reporter in tin^{346/ED6058} mutant embryos (S11 Fig). Both Alk protein and reporter gene expression, could be observed in the dorsal epidermis and amnioserosa at stage 10/11 and in the epidermis at stage 14 (S11 Fig), indicating that regulation by Tin is not critical for Alk expression outside the VM. In contrast, Alk and Alk^{EB9-lacZ} reporter gene expression as observed in the VM of control embryos (S11 Fig) was not observed. However, this analysis was inconclusive since it is difficult to address if, and to which extent, VM formation proceeds in tin mutant embryos. Bin and Bap TFs are known to have a critical function during Drosophila VM development [12, 15]. In our initial experiments we were unable to see any effect on Alk expression on ectopic expression of either Bin or Bap alone in the epidermis employing en-GAL4 as driver (S12 Fig), however this may reflect a lack of tissue competence in our experimental approach. Therefore, we analyzed both Alk protein and Alk^{EB9-lacZ} expression in bin and bap mutants focusing on VM expression. Although VM development does not proceed normally in either bin or bap mutants, we could observe Alk protein and Alk^{EB9-lacZ} expression in the VM in both cases (S13B–S13C’ Fig). We next investigated Alk^{EB8-lacZ} expression, which was reduced in both bin mutants and bap mutants (S13E–S13F’ Fig). On closer inspection of the Alk^{EB8} region we identified four putative Bap binding sites, which we deleted to create Alk^{EB8AbapBS-lacZ}. Alk^{EB8AbapBS-lacZ} failed to exhibit reporter expression suggesting that Bap may be involved in Alk expression in the VM through binding sites within the Alk^{EB8} region (Fig 9A and 9B). Based on these findings, we analyzed Alk protein levels in Alk^{AOpasBS-10.28.3;bin^{1};BSC374} and Alk^{AOpasBS-10.28.3;bap^{208};ED6058} double mutant backgrounds to test whether Alk expression was affected in a combinatorial manner. We observed a strong reduction of Alk expression in the VM of both Alk^{AOpasBS-10.28.3;bin^{1};BSC374} mutants (Fig 9C and 9D; quantified in Fig 9G) and Alk^{AOpasBS-10.28.3;bap^{208};ED6058} mutants.
These results suggest that additional factors, including Bin and Bap, contribute to regulate $Alk$ expression in the VM through the $AlkEB8$ region of the $Alk$ locus (Fig 9I).

**Discussion**

In this study we report the identification of $Alk$ cis-regulatory elements and TF binding sites that control the expression of $Alk$ during embryogenesis. We have been able to identify regions that regulate transcription of $Alk$ in the AS, the VM and the epidermis. We further identify the Opa TF as well as Bin and Bap as regulators of $Alk$ transcription in these tissues during embryogenesis. Taken together our results shed light on the regulatory mechanisms...
controlling *Alk* transcription and identify important *cis*-regulatory sequences required for regulation of *Alk* gene expression.

**Transcription from the *Alk-RB* promoter is essential for *Alk* expression in the visceral mesoderm**

The importance of Jeb/*Alk* signaling *in vivo* in the embryonic VM for FC specification is well established [2–5]. From this earlier work we know that activated *Alk* in the VM triggers not only transcriptional activation but also post-translational modifications that promote the specification of the FC fate [3–6, 11]. In contrast, very little is known about factors that mediate *Alk* transcriptional regulation. In this study we aimed to identify CRMs and TFs important for *Alk* transcription. The *Alk-RA* and *Alk-RB* transcripts encode the same protein, but differ in their 5′ non-coding regions which employ alternative promoters [1]. This potentially allows differential expression of the *Alk-RA* and *Alk-RB* mRNA isoforms both temporally and spatially. Such regulation has been described previously for genes such as the *Drosophila* DOA kinase [27] and the BBG PDZ-protein [28], among others. Embryos in which the promoter of the *Alk-RB* isoform has been disrupted fail to express detectable *Alk* protein in the VM, AS and epidermis, and exhibit an *Alk* loss of function phenotype, revealing that this promoter is critical for *Alk* expression in these embryonic tissues. However, expression of *Alk* in the embryonic CNS is not compromised by the removal of the *Alk-RB* promoter and upstream sequences, suggesting that CNS expression of *Alk* is independent of the VM, AS and epidermal enhancers identified here. Taken together, our results indicate a critical requirement for *Alk-RB* expression to ensure sufficient *Alk* protein levels in the VM for signaling and founder cell specification, as well as for *Alk* expression in the AS and epidermis where the function of *Alk* is currently uncharacterized.

**Enhancer elements upstream of the *Alk* locus regulate expression in the amnioserosa, visceral mesoderm and epidermis**

Previous reports have studied sequences within the *Alk* locus either by reporter activity assays [1, 17] or ChIP-on-chip analyses [16, 17]. Our analysis of reporter activity has identified regions upstream of *Alk* that are active in the AS, VM and epidermis. These coincide temporally with *Alk* protein expression, allowing us to define *Alk* VM, AS and epidermal enhancers located proximal to the *Alk-RB* promoter. High-throughput Y1H screens performed in this study identified a number of TFs that potentially bind to and regulate these regions of the *Alk* locus. In addition, a genome-wide ChIP-on-chip screen for mesodermal TFs occupancy identified a CRM upstream of the *Alk* locus that is active during mesoderm development [16]. This CRM maps to 2R:16,639,969..16,640,341 (relative to Dmel_Release_6 sequence assembly) and was described to be bound by mesodermal TFs including Bin, Bap, Mel2 and Tin and Twi [12, 15, 16, 29, 30]. However, none of these factors were found in our Y1H analysis. This may reflect additional requirements for binding of some TFs, which would preclude their identification by Y1H, such as heterodimerization with co-factors or post-translational modifications. Interestingly, homozygous mutants for *bin* and *bap* still express *Alk* protein in the VM, suggesting that while they may be involved in the modulation of *Alk* expression, additional factors are also important in the regulation of *Alk* expression in the VM.

One such factor could be the NK-4/msh-2 TF Tinman (Tin) which has been previously reported to bind CRMs at the *Alk* locus [16, 17]. Indeed, expression of *Alk* in the VM is affected in *tin* mutant embryos ([17], this study) however it is not clear if this occurs due to direct regulation of *Alk* expression by Tin or a general lack of induction of the VM lineage. Moreover, our analysis of *Alk^{AOpabS};bap* double mutants uncovers a severe decrease in *Alk*
protein in the VM suggesting only a minor direct contribution of Tin. Interestingly, *opa* has been reported to be directly regulated by Tin during heart development [17, 31] and Tin is critical for the expression of two key VM TFs *bin* and *bap* [4, 15]. Therefore it is likely that the importance of Tin for *Alk* expression relies on its activating potential for these *Alk*-regulating TFs. Interestingly, loss of *tin* does not affect *Alk* expression in the epidermis.

Reporter gene expression analyses suggest the VM *Alk* enhancer is located upstream of the *Alk*-RB isoform, in agreement with previously reported *Alke301-lacZ* reporter spanning 1,984 bp (Fig 1A; S1A Fig) [17] and the 547 bp MesoCRM-880 [16] that both cover the *AlkEB9* region. Our data suggests that *Alk*-RB expression can be activated through an upstream enhancer that is bound by Opa located within *AlkEB9*. We were also able to identify additional nearby enhancer elements in *AlkEB8* that integrate information from factors such as Bin and Bap that are critical to ensure precise and robust VM expression of *Alk*. Taken together with the earlier ChIP analyses from the Furlong and Frasch groups, our data suggest that Opa, along with mesodermal TFs such as Bin, Bap, Mef2 and Tin and Twi function in a combinatorial manner to drive robust expression of *Alk* in the VM (Fig 9I).

**Opa activates *Alk* transcription through the *AlkEB9* enhancer**

Our efforts to identify novel TFs involved in *Alk* transcriptional control by *in vitro* Y1H assay resulted in a cluster of TFs potentially binding the *AlkEB9* sequence. Of those TF hits for which UAS-transgenes were available to test, only Opa was observed to induce cell autonomous expression of *Alk* when ectopically expressed. *opa* is a pair-rule gene [32] that encodes a zinc finger protein important during embryonic segmentation and midgut formation [26, 33, 34], as well as adult head morphogenesis by direct regulation of *decapentaplegic* (*dpp*) transcription [23, 35]. *opa* transcript is expressed in a spatially and temporally dynamic pattern, starting from stage 5 in a broad expression domain and from stage 11 onwards in two discrete domains in the VM corresponding to the first and third midgut constrictions [26, 33].

While Opa plays a role in the differentiating midgut musculature, with *opa* mutants exhibiting an interrupted VM unable to form midgut constrictions during embryogenesis [26], its role during segment formation presents a challenge when attempting to decipher the contribution of this TF more precisely. One component of this may be the regulation of *Alk* by Opa shown here. While we observed that *opa* mutants display lower levels of *Alk* protein in the VM, Jeb/Alk signaling is not abrogated, suggesting that while reduced, *Alk* protein levels are not reduced to levels under the threshold critical to drive Alk signaling. The lack of a critical role for Opa in the VM expression of *Alk* may reflect the importance of *Alk* signaling in this tissue for survival of the fly, where a more complex network of TFs may be employed to ensure rigorous *Alk* expression.

**A role for Bin and Bap in regulation of *Alk* transcription in the VM**

Additional VM enhancer elements 5′ of *AlkEB9* in the *Alk* locus are regulated in part by Bin and Bap, two TFs that are critical for VM development. Thus multiple partially redundant enhancer regions are employed to safeguard VM expression of *Alk*, a phenomenon that has been observed in numerous genes expressed in the *Drosophila* embryonic muscle [36]. Moreover, while we have tested the role of Opa and the Opa binding sites in the *AlkEB9* region of the *Alk* locus in this work, we have done so under standard laboratory conditions, and as a result have not tested whether either Opa itself, *AlkEB9* or *AlkEB8* VM enhancers may play an increasingly critical role in *Alk* expression in more demanding environmental conditions, as it has been described for some *Drosophila* loci [37]. Although *Alk* is expressed in *bin* and *bap* mutants, our experiments combining deletion of the Opa binding region in *Alk* in a *bin*
or bap mutant background suggest a combinatorial role for Bin, Bap and Opa driving VM expression of Alk [12–15]. Opa, Bin and Bap potentially act in combination with other TFs to control Alk transcription in the VM, as has been described for sloppy paired-1 (slp1) activation in the somatic blastoderm in response to Opa and Runt [38]. In addition to direct regulation of Alk expression, Opa may also impact Alk expression via indirect mechanisms during embryogenesis.

Further complexity arises when the regulation of opa itself in the VM is considered. It is known that Dpp signaling restricts the VM spatial expression pattern of opa to PS6-8, with dpp mutants showing continuous opa expression throughout the VM [26]. Opa is also known to regulate dpp expression during adult head development [23]. In addition, opa is broadly expressed in the mesoderm at stage 6 potentially driving Dpp signaling. The Dpp mesodermal response consists of up-regulation of tin and bap, important regulatory genes in the dorsal mesoderm that essentially contribute to the specification of the VM [12, 39]. Similarly, Alk activity, the FoxF forkhead domain TF Bin and the Tbx1 Org-1, are also critical factors for expression of dpp in the VM and subsequent activation of Mad signaling in the midgut endoderm [40, 41]. Moreover, loss of org-1, whose expression is maintained by Alk signaling in the VM, results in decreased opa VM expression [4, 41], revealing a complex interplay of regulation where both Alk and Opa control each other’s expression in a spatially and temporally regulated manner.

Surprisingly, in addition to a non-essential role for Opa in the regulation of Alk transcription in the VM, in this work we have been able to identify a critical role for Opa in Alk expression in the AS and epidermis. Here, in contrast to the VM, Opa appears to be required and sufficient to drive Alk expression, although the functional significance of Alk in these tissues remains uncharacterized. Expression of the AlkEB9-lacZ reporter and derivatives in which the Opa binding sites have been mutated indicate that Opa has an important function in Alk transcription through the predicted Opa BS. This is supported by the absence of detectable Alk protein in the AS and epidermis of Alk\(^{\Delta\text{OpaBS}}\) mutants, where the Opa binding sites within the AlkEB9 enhancer have been deleted. Given that Alk\(^{\Delta\text{OpaBS}}\) mutants are viable, it may be that Alk signaling is employed in a small population of non-essential cells that remain to be identified. Further work will be required to characterize the role of Alk in this context.

We have focused here on the regulation of Alk expression during embryonic development, however, Alk is also observed in larval and adult stages. Although Alk signaling does not seem to be critical for viability post-embryogenesis, a number of important roles in the nervous system have been described [42–47]. While we have not investigated the role of Opa, Bin or Bap in Alk expression at these other stages, nor in the CNS in this study, this would certainly be of interest to address in future experiments.

**Materials and methods**

*Drosophila* stocks and genetics

Standard *Drosophila* husbandry procedures were employed. *Drosophila* strains and crosses were maintained on a potato-meal based diet. Crosses were performed at controlled 60% humidity and 25°C conditions. Fly lines used in this study are: UAS-Alk [1], UAS-GFP (Bloomington 4775), UAS-bap.3xHA (FlyORF #F000006), UAS-bin.3xHA (FlyORF #F000281), UAS-jeb [6], UAS-lacZ (Bloomington 1776), UAS-opa [35], UAS-opa\(_{\text{RNAI}}\) (VDRC KK108975), UAS-pnt.P1 (Bloomington 869), UAS-side (Bloomington 9679), Alk\(^{1}\) [2], Alk\(^{10}\) [2], bap\(^{208}\) [12], Df(3R)ED6058 (Bloomington 24140), bin\(^{1}\) (Bloomington 1438), Df(3L)BSC374 (Bloomington 24398), opa\(^{1}\) (Bloomington 3312 and 3222), opa\(^{8}\) (Bloomington 5335), tin\(^{346}\) [12], AlkEl6.5-GAL4 [1], bap3-GAL4 [15], en2.4-GAL4 (Bloomington 30564), prd-GAL4...
(Bloomington 1947), twi.2xPE-GAL4 (Bloomington 2517), HandC-GFP [48], opaΔD246 [26], Opa4opt-lacZ and Opa4opt-KO-lacZ [23]. Alk alleles generated in this study are summarized in S1 Table.

Transgenic flies generated in this study: AlkE4-GAL4, AlkE2.7-GAL4, AlkEB9-GAL4, eve.p: empty-lacZ, AlkEB6-lacZ, AlkEB7-lacZ, AlkEB8-lacZ, AlkEB9-lacZ, AlkB11-lacZ, AlkEB9_OpaBS-lacZ, AlkEB9_OpaKO-lacZ, AlkEB8ΔBapBS-lacZ, AlkEB8ΔEB9-lacZ, AlkEB8/EB9+50flank-lacZ and AlkEB8/EB9+100flank-lacZ. Molecular details of the regions covered by these fragments are described in S2 Table. Genomic coordinates refer to the Dmel_Release_6 sequence assembly [49].

**Immunohistochemistry**

Embryos were stained as described [1]. Primary antibodies used were: guinea pig anti-Alk (1:1000 [3]), rabbit anti-β-galactosidase (1:150; Cappel 0855976), chicken anti-β-galactosidase (1:200; Abcam ab9361), mouse anti-Fasciclin III (1:50; DSHB 7G10), rabbit anti-GFP (1:500; Abcam ab290), chicken anti-GFP (1:300; Abcam ab13970), mouse 16B12 anti-HA.11 (1:500; Covance #MMS-101P), rabbit anti-Org-1 (1:1000, this work), sheep anti-digoxygenin-AP fab fragment 1:4000 (Roche). Alexa Fluor®-conjugated secondary antibodies were from Jackson Immuno Research. Embryos were dehydrated in an ascending ethanol series before clearing and mounting in methylsalicylate.

Images were acquired with a Zeiss LSM800 confocal microscope or Axiocam 503 camera, processed and analyzed employing Zeiss ZEN2 (Blue Edition) imaging software. For analysis of protein levels, the laser, pinhole and PMT settings were adjusted on control siblings subsequently employed for imaging of mutant embryos.

Fluorescence intensity measurements were quantified using Zeiss ZEN2 (Blue Edition). In brief: mean fluorescence values were acquired from regions of interest (ROI), corresponding to the VM or epidermis (Alk staining) selected in confocal sections of stage 11 embryos. This mean fluorescent intensity was corrected using a background ROI chosen from a non-stained area. Measurements were taken from 10 embryos per sample analyzed. For statistical analysis we performed a one-way ANOVA using GraphPad Prism 6 software, where n.s. stands for non-significant, ***p≤0.001 and ****p≤0.0001. All plots are visualized as mean ±S.D.

**Generation of Org-1 antibodies**

Recombinant N-terminal Org-1 protein was produced from pET30a—Org-1-N as generated by [50] was purified by His affinity chromatography and injected into rabbits for antibody generation (Genscript).

**In situ hybridization**

For in situ hybridization, fragments of Alk, gprs, CG5065 and opa were amplified from genomic DNA with the primer combinations shown in S3 Table. PCR products were cloned into the dual promoter PCRII TOPO vector (Invitrogen) and used as template to generate DIG-labeled in situ probes with SP6/T7 polymerases (Roche). In situ hybridization of antisense probes to embryos was carried out as previously described [51]. Samples were mounted in in situ mounting media (Electron Microscopy Sciences).

**High-throughput yeast one-hybrid screening**

pMW2-vectors containing the different Alk putative CRMs were generated by regular cloning techniques (primer combinations shown in S4 Table) and integrated into the yeast genome as
Each DNA bait yeast strain was then transformed with a library of 647 Drosophila TFs fused to GAL4. Interaction was assessed by growing transformant yeast strains on selective plates followed by data analysis as previously described [18]. Briefly, selective growth of diploid yeast colonies was analyzed by the Matlab-based image-analysis program TIDY which quantifies bright spots, representing yeast colonies to the dark background. For every biological replicate in the screen, each bait-TF interaction was analyzed in four technical replicates resulting in quadrants of yeast colonies as shown for AlkEB9 DNA bait in the results.

**Generation of transgenic flies**

For generation of lacZ reporter flies, DNA sequences of for AlkEB6 to AlkEB11 were PCR amplified (S4 Table) and cloned into the eve.p-lacZ.attB vector [52]. In addition, the AlkEB9 DNA bait was cloned into pPT-GAL vector (1225, DGRC) to generate the AlkEB9-GAL4 construct. DNA sequences for AlkEBδAbapBS-lacZ, AlkEB9_OpaBS-lacZ and AlkEB9_OpaKO-lacZ were assembled by Genscript and cloned into eve.p-lacZ.attB vector for further PhiC31 directed genome integration. For generation of AlkE4-GAL4 and AlkE2.7-GAL4 constructs, DNA genomic regions covering 2R:16,638,503..16,642,495 and 2R:16,638,510..16,640,834, respectively, were cloned into pCaSpeR-DEST6 (1032, DGRC) by the Gateway system (primer combinations in S4 Table). Constructs were sequenced (GATC Biotech) and injected into w1118 flies, except for attB constructs which were injected into Bloomington 24482 and 24485, for PhiC31 directed integration at 51C and 68E respectively (BestGene Inc.).

**Generation of Alk mutants**

Deletions within the Drosophila Alk enhancer region were generated with CRISPR/Cas9 [53]. The sgRNA targeting sequences used (listed in S1 Table) were cloned into pBFv-U6.2 expression vector (Genome Engineering Production Group at Harvard Medical School). Constructs expressing sgRNA were injected into vasa (vas)-Cas9 (Bloomington 51323) embryos by BestGene Inc. Screening of deletion events was performed by PCR and further sequencing (GATC Biotech). For additional complementation tests we employed balanced Alk10 or Df(2R) Exel7144 flies.

**Electrophoretic Mobility Shift Assay (EMSA)**

DNA coding sequence of opa was synthesized (Genscript) in frame with carboxy-terminal OLLAS and 6xHis tags and cloned into the pcDNA3.1(+) mammalian expression vector. Binding of Opa to the AlkEB9 was analyzed by a DNA binding assay on dsDNA oligonucleotides with cell lysates from HEK-293F cells expressing Opa-OLLAS. Binding reactions were performed as described in [54] containing 10 mM Tris-HCl (pH 8.0), 25 mM KCl and 1 mM DTT, 1 μg poly-dIdC (Sigma-Aldrich), 2.5% glycerol, 0.05% Triton X-100, 0.2 mM MgCl2 and the indicated 3’-end biotin labelled probe. After 20 min incubation at room temperature, reactions were separated on a 6% native TBE-PAGE in 0.5x TBE buffer at 100V. DNA was transferred to nylon+ membranes (Amersham), UV cross-linked to the membrane and detected by Chemoluminescence Nucleic Acid Detection Module (Pierce) according to manufacturer’s indications. Competition assay was performed by addition of 100 fold molar excess of unlabeled competitor DNA to the reaction mix. Wild-type probes used for band shift experiments were Opa_SELEX and Opa_JASPAR. Mutated version were made according to for Opa_SELEX mutant [23], and in a similar manner for Opa_JASPAR mutant. All four EMSA probe sequences are shown in S3 Table.
Chromatin immunoprecipitation

Chromatin was prepared from approximately 100 mg of pooled collections of fixed 3–4 hour embryos. The embryos were homogenized for 1 min in 10 mM EDTA and 50 mM Tris (pH 8.1). After addition of SDS to a final concentration of 1% and incubation on ice for 10 min, glass beads (150–200 μm) were added and the homogenates were sonicated to give sheared chromatin preparations with an average DNA size of 300–400 bp. Chromatin immunoprecipitation was performed largely as described previously [55] using an affinity-purified anti-Opa antibody raised against a truncated recombinant protein spanning from amino acids 125–507 of Opa, a region containing the DNA-binding zinc-fingers at a concentration of 0.5 μg/ml with 100 μg of chromatin in 1 ml of 0.01% SDS, 1% TritonX-100, 1 mM EDTA, 20 mM Tris, pH 8, 150 mM NaCl and 1x Protease Inhibitor Cocktail (Roche). After overnight incubation of the chromatin and antibody at 4˚C, the mixture was incubated with Protein-A Agarose (Millipore) for 2 hours at room temperature, followed by low-salt, high salt and LiCl washes as used in the Chromatin Immunoprecipitation Assay Kit (Upstate Biotechnology). After heat reversal of protein-DNA crosslinks, protein digestion, phenol chloroform extraction and purification of the nucleic acids by ethanol precipitation the amount of recovered DNA was quantified using qPCR and a standard curve generated for each primer pair with a sample of nucleic acid purified from the input chromatin. The control primer pair produces a 115 bp amplicon located 12.4 kb upstream of odorant receptor 42b, a region devoid of modEncode hallmarks of cis-regulatory DNA sequences. The DESE-Opa primer pair produces a 209 bp amplicon from a central region of the slp1 DESE enhancer that requires Opa for expression [56]. The Alk primer pair produces a 140 bp amplicon that extends from 21 bp downstream of the SELEX_OpaBS to 53 bp upstream of the JASPAR_OpaBS. The ChIP values that are reported are percent precipitation relative to input DNA with error bars representing the mean ± S.D. from three technical replicates of the qPCR. The sequences of the primers are summarized in S3 Table and are as follows: Or42b forward: 5’ TCAAGCCGAACCCTCT AAAAT 3’, Or42b reverse: 5’ AACGC CAACAAACAGAAAATG 3’, DESE-Opa forward: 5’ TGCCGTTCGAGTCCT TTATT 3’, DESE-Opa reverse: 5’ CGGAGATCGGAAGGT TAGTG 3”, Alk-OpaBS forward: 5’ TTGTGCG TTTCACCAATCG 3’, Alk-OpaBS reverse: 5’ CGGACTAGCCACATCGAAC 3’.

Supporting information

S1 Fig. Schematic overview of the Alk locus summarizing this study. (A) Schematic representation of the Alk locus and its exon-intron structure, coding sequences shown in white. GAL4 lines covering the 5’ region of the Alk locus are shown as blue lines. The MesoCRM-880 and AlkE301 CRMs identified in previous ChIP analyses are depicted as grey lines. DNA baits subjected to Y1H analysis—region shown in red dashed lines—are depicted as black lines (AlkEB6 –AlkEB11). A 2 kb close up window (shaded yellow) indicates the region shown in B and C. (B) Overview of lacZ reporters generated covering AlkEB8 and AlkEB9 (light blue lines). Predicted binding sites for mesoderm TFs (pink), Opa (red) and Bap (grey) are indicated. (C) Summary of the different deletions generated by CRISPR/Cas9 genome editing (dashed lines) and employed in this study. (TIF)

S2 Fig. Alk regulation in the presumptive Amnioserosa (AS). (A) Alk mRNA is observed in the dorsal most region of stage 6 Drosophila embryos, AS. (B) Left panel: AlkE6.5-GAL4 drives reporter expression in the AS (red), overlapping with Alk protein (green), in stage 10 embryos. Right panel: enlargement of boxed area, showing Alk protein (green) and lacZ expression (red) in AS cells. (C) AlkEB8-lacZ does not drive reporter expression in the AS (lacZ activity in red,
Alk protein in green). (D) AlkB9-lacZ drives reporter expression in the AS (lacZ activity in red, Alk protein in green). Scale bars: 50 μm.

(TIF)

S3 Fig. Expression pattern of genes neighboring the Alk locus. (A) gprs transcripts are detected at later stages of embryogenesis in the developing CNS, with strong expression detected in the ventral midline. (B) CG5065 transcripts were also only observed at later stages of embryogenesis in the foregut, hindgut and developing CNS. Scale bars: 50 μm.

(TIF)

S4 Fig. Alk mRNA is absent in the VM and epidermis of the Alk-RB mutant, but is unaffected in the embryonic CNS. AlkB1,22 mutants lack mRNA in the VM at (B, stage 9) when compared to controls (A). Alk mRNA levels are unaffected in the CNS of AlkB1,22 mutant embryos (B, stage 16, compare with A). Scale bars: 50 μm.

(TIF)

S5 Fig. Quantification of AlkB8-lacZ and AlkB9-lacZ in the VM. Both AlkB8-lacZ and AlkB9-lacZ are active in the VM, although AlkB8 displayed significantly less activity when compared to AlkB9. Degrees of significance are denoted by ****p<0.0001 (n = 10 animals per genotype).

(TIF)

S6 Fig. Ectopic expression of candidate TFs controlling Alk expression. (A) Stage 14 embryos display Alk protein in a segmented fashion in epidermis (asterisks), while prd-GAL4 driver is active in every second cluster of Alk positive epidermal cells (dashed box indicates area of close-up). (B) Ectopic expression of side under prd-GAL4 control does not lead to any changes in Alk protein in epidermis (asterisks; dashed box indicates area of close-up). (C) Overexpression of pnt.P1 with prd-GAL4 leads to a loss of Alk positive cells in epidermis (asterisks; dashed box indicates area of close-up). Scale bars: 50 μm and 10 μm (embryo and close ups, respectively).

(TIF)

S7 Fig. Quantification of different reporter lines activity. AlkB9-lacZ, AlkB9_OpaBS-lacZ and AlkB9_OpaKO-lacZ reporter activities were quantified in both the VM and the epidermis. AlkB9-lacZ and AlkB9_OpaBS-lacZ show a similar expression level in the epidermis, while AlkB9_OpaKO-lacZ expression was significantly reduced. In the VM, expression of AlkB9-lacZ was stronger than that of AlkB9_OpaBS-lacZ, while mutation of the Opa binding sites in AlkB9_OpaKO-lacZ resulted in a further reduction of activity. Degrees of significance are denoted by n.s. (not significant), *** (p<0.001) and **** (p<0.0001).

(TIF)

S8 Fig. Opa mRNA expression pattern and role in Alk signaling. (A) opa mRNA is expressed at high levels in a broad domain at stage 5. At later stages opa transcript is observed as 14 stripes of stronger expression alternating with stripes of weaker expression (stage 9). At stage 12, opa expression in discrete clusters of cells is observed in the VM (arrowheads), continuing at stage 14 where opa expression appears as two broad bands in the VM corresponding to parasegments 3–5 and 9–12 (arrowheads) and continuing to late embryogenesis. Embryos are oriented anterior left, dorsal up. (B, C) Org-1 (red) is observed in FCs nuclei in response to Jeb/Alk signaling (insets show area of close up). Levels are reduced, although still present, in opa⁺/opa⁻ mutants when compared with controls. Alk is shown in green. (B’, C’) Org-1 shown in white. (D, E) Similarly, HandC-GFP (blue) reflects Alk (green) signaling activity, but is not affected upon Opa overexpression in the VM with the bap3-GAL4 driver (insets show area of
close up). (D', E') Org-1 shown in white, (D'', E'') HandC-GFP shown in white. Stage 11 embryos are shown in B-E''. Scale bars: 50 μm.

(S9 Fig) Functional analysis of additional non-lethal CRISPR/Cas9 generated deletion mutants in the Alk locus. (A, A') AlkΔmesoBS mutants do not show reduced levels of Alk protein in either the VM or epidermis (A, quantified in B). Furthermore, stage 16 homozygous mutant embryos show normal FC specification (inset, stage 11 embryo) and a chambered gut (A', stage 16 embryo). (B) Quantification of Alk protein levels in AlkΔmesoBS mutants (n.s.–not significant; n = 10 animals per genotype). Scale bars: 50 μm.

(S10 Fig) Reporter activity analysis of the overlap region between AlkEB8 and AlkEB9. (A-C) lacZ reporters corresponding to the overlapping region between AlkEB8 and AlkEB9 were analyzed for reporter activity (schematically depicted in S1 Fig). None of the three transgenic sequences containing increasing portions of the overlapping region between AlkEB8 and AlkEB9, namely EB8'-9-lacZ (111 bp), EB8'-9+50flank-lacZ (211 bp) and EB8'-9+100flank-lacZ (311 bp) exhibit any detectable lacZ reporter activity during embryogenesis. Upper panels show lacZ reporter expression in white. Lower panels show a merged image of Alk protein shown in green and lacZ reporter expression in red. Scale bar: 50 μm.

(S11 Fig) AlkEB9-lacZ expression in tin mutant background. (A-A') Expression of AlkEB9-lacZ in stage 11 tin/ED6058 mutant embryos. Lateral view of epidermal Alk (red) and β-gal (green) (A). Deeper view reveals no detectable Alk (red) or lacZ(green) expression (A'). (B-B') Stage 14 tin/ED6058 mutant embryos do not exhibit VM structures (B'), however, both expression of AlkEB9-lacZ (green) and Alk protein (red) is visible in the epidermis (B). Scale bar: 50 μm.

(S12 Fig) Ectopic expression of either Bin or Bap does not drive ectopic Alk expression. (A-C) Ectopic expression of either bap (B) or bin (C) in the epidermis with en-GAL4 does not result in a detectable increase in Alk protein levels. Alk protein shown in red, GFP in green (A), anti-HA in green (B, C). Scale bar: 50 μm.

(S13 Fig) Bin and Bap are required for full AlkEB8-lacZ activity. (A-C') Expression of AlkEB8-lacZ in the VM is not altered in bin''/BSC374 or bap''/ED6058 embryos (arrowheads). (D-F') In contrast, expression of AlkEB8-lacZ is mildly reduced in bin''/BSC374 animals and undetectable in bap''/ED6058 embryos (arrowheads). Alk protein is shown in green, lacZ reporter expression in red, FasIII shown in blue. Scale bar: 50 μm.

(S1 Table) Summary of CRISPR/Cas9 mutants generated in this study. Molecular details of each mutant and characterization of their Alk expression patterns are included.

(S2 Table) Summary of transgenic Drosophila generated in this study. Molecular details are included.

(S3 Table) Oligonucleotide sequences employed for in situ, EMSA and ChIP analysis.
S4 Table. Primer combinations used to clone DNA-baits into either pMW2 for yeast one-hybrid screening or eve.p-lacZ.attB for reporter analysis.

(XLSX)

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Author Contributions
Conceptualization: PMG FH MF MLH YI KP GW GV DP JPG KH BD RHP.
Formal analysis: PMG FH MLH YI.
Funding acquisition: JPG BD RHP.
Investigation: PMG FH MF MLH YI KP GW GV DP.
Methodology: PMG JPG KH BD.
Project administration: JPG BD RHP.
Supervision: GW JPG KH BD.
Visualization: PMG FH KP GW JPG BD RHP.
Writing – original draft: PMG FH MF KP GW DP KH BD RHP.
Writing – review & editing: PMG FH MF MLH YI KP GW GV DP JPG KH BD RHP.

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