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Klumpfuss controls FMRFamide expression by enabling BMP signaling within the NB5-6 lineage

María Losada-Pérez1, Hugo Gabilondo1, Isabel Molina1, Enrique Turiegano1, Laura Torroja1, Stefan Thor2 and Jonathan Benito-Sipos1,*

SUMMARY
A number of transcription factors that are expressed within most, if not all, embryonic neuroblast (NB) lineages participate in neural subtype specification. Some have been extensively studied in several NB lineages (e.g. components of the temporal gene cascade) whereas others only within specific NB lineages. To what extent they function in other lineages remains unknown. Klumpfuss (Klu), the Drosophila ortholog of the mammalian Wilms tumor 1 (WT1) protein, is one such transcription factor. Studies in the NB4-2 lineage have suggested that Klu functions to ensure that the two ganglion mother cells (GMCs) in this embryonic NB lineage acquire different fates. Owing to limited lineage marker availability, these observations were made only for the NB4-2 lineage. Recent findings reveal that Klu is necessary for larval neuroblast growth and self-renewal. We have extended the study of Klu to the well-known embryonic NB5-6T lineage and describe a novel role for Klu in Ap cluster specification in general and particularly in Ap4 neuron differentiation, indicating that Klu is necessary for larval neuroblast growth and self-renewal. We have extended the study of Klu to the well-known embryonic NB5-6T lineage and describe a novel role for Klu in the Drosophila embryonic CNS. Our results demonstrate that Klu is expressed specifically in the postmitotic Ap4/FMRFa neuron, promoting its differentiation through the initiation of BMP signaling. Our findings indicate a pleiotropic function of Klu in Ap cluster specification in general and particularly in Ap4 neuron differentiation, indicating that Klu is a multitasking transcription factor. Finally, our studies indicate that a transitory downregulation of klu is crucial for the specification of the Ap4/FMRFa neuron. Similar to WT1, klu seems to have either self-renewal or differentiation-promoting functions, depending on the developmental context.

KEY WORDS: Drosophila, Klumpfuss, Terminal differentiation, BMP signaling, Neuropeptidergic cell identity, FMRFa

INTRODUCTION
The nervous system contains a daunting number of cells and a vast diversity of cell types. Neurons differ from each other in many ways, including morphology and the type of neurotransmitters/neuropeptides, receptors and ion channels that they express. Each neuronal subtype needs to be generated at the correct place, precise time, and in the appropriate numbers. The combined effect of vast cell numbers, great diversity and strict fidelity of pattern constitutes the very basis for the enormously complex functions of the nervous system, such as homeostasis, learning/memory and behavior. Understanding neuronal subtype specification continues to be one of the fundamental challenges in neurobiology. Drosophila embryonic ventral nerve cord (VNC) is an important model system for addressing basic mechanisms of nervous system development. It is becoming increasingly clear that most developmental mechanisms are highly conserved across the animal kingdom, and findings in less complex models have been crucial for elucidating the molecular and genetic mechanisms that control nervous system development in higher animals.

To generate neural diversity at the precise time and place required, while establishing the correct connections, Drosophila embryonic developing VNC needs to orchestrate the expression of large numbers of regulatory genes with great temporal and spatial precision. The involvement in neural subtype specification of a set of genes that encode transcription factors that are expressed within most, if not all, embryonic neuroblast (NB) lineages, has been broadly described. Some of these genes are components of the well-known temporal gene cascade, which controls temporal competence changes within NB lineages, generating different cell types at different time points. Nevertheless, the function of other widely expressed transcription factors has only been described within specific NB lineages. Hence, it remains unknown to what extent their functions operate in other lineages. Klumpfuss (Klu) is one such transcription factor. klu encodes a protein with four zinc-finger motifs of the C2H2 type, three of which are homologous to those of the proteins of the EGR transcription factor family whereas the fourth is highly homologous to the divergent zinc-finger of mammalian Wilms tumor 1 (WT1) (Klein and Campos-Ortega, 1997). Klu is expressed in most, if not all, embryonic neuroblasts and has been found in GMC-2 of several lineages. Klu has been shown to prevent GMC4-2b from adopting the GMC4-2a fate by repressing even skipped (eve) expression in GMC4-2b, and loss of klu expression leads to a duplication of GMC4-2a fate. Although it is known that Klu does not affect the progeny of GMC4-2a, the role of Klu in later born ganglion mother cells (GMCs) in the NB4-2 lineage has not been examined (Klein and Campos-Ortega, 1997; McDonald et al., 2003; Yang et al., 1997). From these studies it was concluded that klu functions within embryonic NB lineages to ensure that each GMC in a lineage acquires a different fate. However, owing to the limited availability of lineage markers, these observations were made only for the NB4-2 lineage. Recent findings pinpoint the importance of Klu as a regulator of self-renewal in larval brain neuroblasts, as overexpression of Klu results in the formation of transplantable brain tumors (Berger et al., 2012; Xiao et al., 2012). Klu is necessary for the maintenance of type I and II larval brain neuroblasts, as klu mutant larvae show progressive loss of both types of neuroblasts due to premature differentiation (Berger et al., 2012; Xiao et al., 2012).

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We are taking a comprehensive molecular and genetic approach to understanding neuronal subtype specification, using the *Drosophila* embryonic thoracic neuroblast 5-6 (NB5-6T) as a model. This neuroblast can be readily identified by the specific expression of ladybird early (K) (lbe(K)) reporters (Baumgardt et al., 2009; De Graeve et al., 2004), and is generated in each of the six thoracic VNC hemisegments. Each NB5-6T produces a mixed lineage of 20 cells (Baumgardt et al., 2009). At the end of its lineage, NB5-6T generates directly, without a GMC intermediate, a set of four interneurons, denoted the ‘Ap cluster’, which are defined by expression of the LIM-homeodomain transcription factor Apterous (Ap; mammalian Lhx2a/b) and the transcription co-factor Eyes absent (Eya; mammalian Eya1-4) (Lundgren et al., 2005; Miguel-Aliaga and Thor, 2004). The birth order of each Ap neuron is stereotyped, and the number of the neuron refers to its birth order.

The four Ap neurons can be further subdivided into three neuronal subtypes: the Ap1/Nplp1 and Ap4/FMRFa neurons, which express the Nplp1 and FMRFamide [also known as FMRFamide-related (Fmr1) – FlyBase] neuropeptides, respectively, and two ‘generic’ Ap cluster neurons, herein denoted Ap2 and Ap3 (Fig. 1A,B) (Baumgardt et al., 2009; Baumgardt et al., 2007; Benveniste et al., 1998; Park et al., 2004).

To further understand the development of this lineage and the specification of the Ap neurons, we have conducted a ‘targeted screen’ (Gabilondo et al., 2011) of genes expressed in the VNC (Brody et al., 2002) that alter the FMRFa pattern when mutated. One of the mutants identified in this screen by loss of FMRFa expression was *klu*. Here, we identify a novel role for Klu in the *Drosophila* embryonic CNS. Our results demonstrate that Klu is expressed specifically in the Ap4/FMRFa neuron, where it selectively controls BMP signaling by regulating BMP type I receptors. Our results indicate a pleiotropic function of Klu in Ap cluster specification in general and particularly in Ap4 neuron differentiation, demonstrating that Klu is a multitasking transcription factor. Finally, our studies also indicate that a transitory differentiation, demonstrating that Klu is a multitasking cluster specification in general and particularly in Ap4 neuron receptors. Our results indicate a pleiotropic function of Klu in Ap cluster neurons, herein denoted Ap2 and Ap3 (Fig. 1A,B) (Baumgardt et al., 2009; Baumgardt et al., 2007; Benveniste et al., 1998; Park et al., 2004).

**MATERIALS AND METHODS**

**Fly stocks**

Fly stocks were raised and crossed to generate UAS-flies. The two transgenic lines that represent the genetic background used for all crosses were the UAS-GFP and UAS-NM-EGFP strains, both obtained from Bloomington (UAS-GFP (Baumgardt et al., 2009); UAS-nm-EGFP (Baumgardt et al., 2009). Mutants were kept over CyO, Act-GFP; CyO, Dfj-EYFP; TM3 Ser, Act-GFP; CyO, twi-Gal4, UAS-GFP; TM3, Sh, Ser, twi-Gal4, UAS-GFP; or TM6, Sh, Th, Dfj-EYFP balancer chromosomes. As wild type, Oregon-R was often used. Unless otherwise stated, flies were obtained from the Bloomington *Drosophila* Stock Center.

**Immunohistochemistry**

Antibodies used were: rabbit anti-Klu (1:1000) [provided by X. H. Yang (Yang et al., 1997)]; guinea pig anti-Col (1:1000), guinea pig anti-Dimm (1:1000), chicken anti-pro-Nplp1 (1:1000), rabbit anti-pro-FMRFa (1:1000) (Baumgardt et al., 2007); mouse anti-Seven up (1:50) (gift of Y. Hiromi, National Institute of Genetics, Mishima, Japan); rabbit anti-pMad (1:500) (4D10, Cell Signaling); mAb Eya10H6 (1:250) (from Developmental Studies Hybridoima Bank). All polyclonal sera were pre-absorbed against pools of early embryos. Secondary antibodies were conjugated with FITC, Rhodamine-RedX or Cy5 and used at 1:500 (Jackson Immunoresearch).

Embryos were dissected in PBS, fixed for 25 minutes in 4% parafomaldehyde, blocked and processed with antibodies in PBS with 0.2% Triton X-100 and 4% donkey serum. Slides were mounted with Vectashield (Vector Labs). Wild-type and mutant embryos were stained and analyzed on the same slide.

**Confocal imaging, data acquisition and staining quantification**

A Zeiss META 510 confocal microscope was used to collect data for all fluorescent images; confocal stacks were merged using LSM software (Zeiss) or Adobe Photoshop CS4. Where appropriate, images were false colored for clarity.

**Statistical methods**

Statistical calculations were performed using SPSS software (v15.0.1; IBM). For statistical significance, Student’s t-test or, in the case of non-Gaussian distribution of variables, a non-parametric Mann-Whitney U-test or χ² test was used. Images and graphs were compiled in Adobe Illustrator.

**RESULTS**

**Loss-of-function of klu abolishes FMRFa expression in the Ap cluster**

To begin dissecting the role of *klu* in the latter part of the NB5-6 lineage, we analyzed the terminal differentiation markers Nplp1 and FMRFa, which are neuropeptides expressed by Ap1 and Ap4, respectively. Whereas Nplp1 expression was largely unaffected in *klu* embryos (Fig. 1F,L,P,Q), we found an almost complete loss of FMRFa in the lateral thoracic areas (Fig. 1G,M,P,Q). Of note, the anterior SE2 FMRFa neurons were completely unaffected (Fig. 1G). Since recent studies showed that loss-of-function of *klu* promotes precocious neuroblast differentiation (Berger et al., 2012; Xiao et al., 2012), we asked whether this putative precocious differentiation could preclude the generation of Ap neurons in the final part of the NB5-6 lineage in *klu* mutants. We utilized Eya and ap-Gal4-UAS-GFP markers, which are specific for Ap neurons. In *klu* mutants, we observed four Ap neurons in over 76% of hemisegments (Fig. 1H,L,N,Q), although occasionally (14%) fewer or more than four Ap neurons developed per hemisegment. However, this alteration was not statistically significant (Fig. 1Q). These results suggested that NB lineage progression is not severely affected in *klu* mutants and hence cannot explain the lack of FMRFa in the Ap4 neuron.

We next analyzed expression of the Dimmed (Dimm) basic helix-loop-helix transcription factor, a ‘master gene’ of neuropeptidergic identity (Allan et al., 2005; Hamaoka et al., 2010; Hewes et al., 2003) that is expressed in the Ap1/Nplp1 and Ap4/FMRFa neurons (Park et al., 2008). We found that *klu* mutants display a significant decrease of Dimm expression in the
Ap cluster (Fig. 1N,P,Q). This reduction was explained by the existence of two Dimm phenotypic groups \((n=72\) hemisegments): 50% of the clusters were wild type (two Dimm cells/cluster) and 50% had an altered number of Dimm cells (5.5% had three Dimm cells/cluster and 44.4% had one Dimm cell/cluster). However, reduction of FMRFa was equal in both groups, demonstrating that there is no correlation between the FMRFa and Dimm phenotypes. These results highlight that loss-of-function of \(klu\) produces a selective defect in the differentiation of the Ap4/FMRFa neuron. However, neither NB lineage progression nor Ap1/Nplp1 specification is affected in \(klu\) embryos.

Klu is highly expressed in neuroblasts and is not rapidly downregulated during differentiation.

Previous studies have shown that Klu is highly expressed in larval brain type I neuroblasts but is rapidly downregulated in GMCs. In type II lineages, Klu is expressed in the neuroblast but is lost from immature intermediate neural progenitors (INPs), reappearing in mature INPs and disappearing again when the GMCs are formed (Berger et al., 2012; Xiao et al., 2012). We mapped Klu expression in detail in the NB5-6T lineage, focusing on the end of the lineage when the Ap cluster is generated, using an antibody specific to Klu protein (Yang et al., 1997).
NB5-6T delaminates at late stage 8. During stages 8 and 9 we detected weak expression of Klu (Fig. 2A,L; data not shown). Klu staining became stronger at stage 10, and robust expression of Klu was evident until the cell cycle exit of NB5-6T at stage 15 (Fig. 2B-H,L). In the Ap cluster (generated from late stage 12 until stage 15), Klu was expressed in the newly born Ap1-Ap3 neurons (Fig. 2E-H,L). However, this expression was lost shortly afterwards. This succinct pulse of Klu expression in these newly born Ap neurons correlates well with an inheritance of Klu protein from the neuroblast, but it is incongruent with the urgency of downregulation of Klu suggested for GMCs (reported both in type I and II lineages) and immature INPs (of type II neuroblasts) (Berger et al., 2012; Xiao et al., 2012). However, the Klu expression profile in Ap4/FMRFa was different. In Ap4, we also observed Klu expression in the newly born neuron (stage 15; Fig. 2H,L), but in this case Klu staining was detected until the end of the stage 17 (J). Finally, at stage 18 hAEL, expression of Klu is no longer evident within the Ap cluster nor in the rest of the VNC (K; not shown). (L) Summary of Klu expression in the NB5-6T lineage. (A-D) gsblacZ+/+; (E-H) lbe(K)-Gal4, lbe(K)-Gal4/UAS-nmEGFP; (I-K) ap-Gal4/UAS-nmEGFP/+.

Fig. 2. Klu expression in the NB5-6T lineage. Expression of Klu (green) within NB5-6T during embryonic development. To the right are lateral view graphic representations of the lineage. Images are composed from confocal stacks, in G and H subdivided into two substacks, from dorsal (up) to ventral (down). Anterior is up in all images. (A-D) NB5-6T is identified as the anterior-most and lateral-most neuroblast within the gsblacZ domain (not shown), as well as by cell size and staining for Deadpan (magenta). (E-H) Alternatively, NB5-6T is identified by reporter gene expression driven from the NBS-6-specific lbe(K) enhancer (magenta). Ap1, Ap2, Ap3 and Ap4 neurons are identified by position and different levels of Cal (not shown) (Baumgardt et al., 2009). (I-K) During later stages, Ap1, Ap2, Ap3 and Ap4 neurons are identified by expression of ap-Gal4-UAS-GFP (magenta) and different levels of Cas staining (not shown) (Baumgardt et al., 2009). (A-H) We detect expression of Klu in the neuroblast from stage 9 until the cell cycle exit of NB5-6T at stage 15. (E-K) In the Ap cluster we find that Klu is expressed in the newly born Ap1-Ap3 neurons (E-H). This expression is extinguished shortly afterwards. However, in the Ap4 neuron we also observed Klu expression in the newly born neuron (H), but in this case Klu staining is detected until the end of the stage 17 (J). Finally, at stage 18 hAEL, expression of Klu is no longer evident within the Ap cluster nor in the rest of the VNC (K; not shown). (L) Summary of Klu expression in the NB5-6T lineage. (A-D) gsblacZ+/+; (E-H) lbe(K)-Gal4, lbe(K)-Gal4/UAS-nmEGFP; (I-K) ap-Gal4/UAS-nmEGFP/+.
Klu overexpression cannot induce dedifferentiation in mature Ap neurons

The klu mutant analysis indicated that loss-of-function of klu does not lead to premature differentiation of the NBS-6 lineage. It has been reported that Klu overexpression causes dedifferentiation of immature INPs within larval type II lineages (Berger et al., 2012; Xiao et al., 2012). However, no detectable phenotype was found in larval type I neuroblasts. We therefore asked whether overexpression of klu could cause dedifferentiation of mature Ap neurons.

We overexpressed klu from different drivers, all of which maintain expression in the postmitotic Ap1-4 cells up to the 18 hAEL stage: worniu-Gal4, prospero-Gal4, elav-Gal4 (Fig. 3C,E,G) and ap-Gal4, which has previously been shown to direct expression to all four postmitotic cells, including the Ap4 neuron (Fig. 3C,H) (O’Keefe et al., 1998). The four Ap neurons were generated and their number was largely unaffected in all genetic combinations of klu overexpression (Fig. 3A; data not shown). To determine whether they retain their terminal differentiation markers we examined the expression of Dimm, Nplp1 and FMRFa. Dimm\(^{+}\) and Nplp1\(^{+}\) neurons were numerically unaffected in these genetic backgrounds (Fig. 3A,D). However, overexpression of klu, similar to its loss-of-function, produced a selective lack of FMRFa in the Ap4 neuron (Fig. 3D-H). These results indicate that FMRFa expression is specifically and completely lost from all Ap4 neurons when klu expression is increased and/or maintained for longer than normal, whereas other markers typical of mature postmitotic Ap neurons (Eya, Dimm and Nplp1) remain intact. We concluded that klu overexpression is unable to induce dedifferentiation in Ap neurons.

Klu is expressed in the newly born Ap4 cell. However, its expression disappears at late stage 17. Together with the overexpression data, this led us to think that klu might need to be downregulated in the Ap4 neuron for its proper specification. To test this hypothesis we misexpressed klu using late drivers: dimm-Gal4, which directs expression into late postmitotic cells, when they acquire the neuropeptidergic fate; and fmrfa-Gal4, a late driver directing expression by the regulatory sequence of the fmrfa gene (Suster et al., 2003). Upon misexpression of klu, FMRFa expression is reduced from the Ap4 neuron for its proper specification. To unravel the role of Klu in the Ap window in more detail, we analyzed the expression of a number of genes crucial for Ap neuron specification. These included the temporal genes castor (cas; mammalian Casz1) and grainy head (grh; mammalian Grhl1-3), as...
well as the subtemporal genes squeeze [sqz; mammalian CIZ (ZnF384)], nab (mammalian Nab/1/2) and seven up (svp; mammalian Nr2f1/2) (Baumgardt et al., 2009; Benito-Sipos et al., 2011; Terriente Félix et al., 2007). We observed no alteration in the expression of these temporal and subtemporal factors (supplementary material Fig. S3A-E,G,H,M,N) in klu embryos. Thus, temporal progression in the later part of NB5-6T development is unaffected in klu mutants. Similarly, expression of the Ap neuron determinant collier [col; also known as knot; mammalian Ebf1-4] (Baumgardt et al., 2009) was unaffected in klu mutants (supplementary material Fig. S3L-N). We conclude that klu does not regulate the Ap neuron determinants cas, grh, sqz, nab, syp and col.

BMP signaling is interrupted in klu mutants

Having analyzed most of the genes identified as being crucial for specification of the Ap4/FMRFa cell fate, none of which was altered in klu mutants, we addressed a possible role for klu in the absence of pMad staining (supplementary material Fig. S1). Why is BMP signaling interrupted in klu mutants? The loss of pMad could reflect an absence of the target gland, the DNH itself. When revealed by btn-lacZ expression (Allan et al., 2003), we found an apparently normal DNH in klu mutants (Fig. 4D). Another possibility is a failure of the Ap4/FMRFa neuron to project its axon to the DNH, with an accompanying failure to receive the TGFβ/BMP ligand Gbb. When we analyzed and quantified the innervation of the Ap4/FMRFa neuron in klu mutants, no statistically significant differences were found between controls and klu mutants in DNH innervation (Fig. 4D,F).

Since the Ap4/FMRFa neuron properly innervates its target gland in klu mutants, we addressed a possible role for klu in the phosphorylation and nuclear translocation of the Smad protein Mothers against dpp (Mad), which can be monitored using antibodies specific to phosphorylated Mad (pMad) (Dorfman and Shiio, 2001; Marquès et al., 2003; Tanimoto et al., 2006). Target-derived BMP signaling is also known to occur in most, if not all, motoneurons in the VNC (Aberle et al., 2002). Hence, we used a specific antibody to pMad to assay for BMP activation in Ap4 neurons in a klu mutant background. Whereas there was no obvious loss of pMad staining in the VNC in general (data not shown), loss of pMad staining was prevalent in the Ap4 neuron (Fig. 4B,E).

In addition, absence of FMRFa was always associated with an absence of pMad staining (100%; Fig. 4B,E). Of note, the lack of FMRFa associated with the overexpression of klu is not explained by an absence of pMad (supplementary material Fig. S1).

Fig. 4. BMP signaling is interrupted in Ap4 neurons in klu mutants. (A,B) pMad staining at 18 h AEL in control (A) and klu mutant (B). pMad staining is lost in klu mutants. Arrowhead (A) indicates Ap4 neuron. (C,D) Overlap of btn-lacZ (red), ap-Gal4>UAS-nmEGFP (green) and FMRFa (blue) at 18 h AEL in control (C) and klu mutant (D). There are no gross differences between controls and klu mutants in DNH innervation. (E) Number of cells expressing FMRFa or showing pMad in controls and klu mutants. n≥35 hemisegments. *P-value compared with control (Chi-square test). Error bars indicate s.d. (F) Quantification of innervation of the Ap4/FMRFa neuron in klu mutants. P>0.001 (Student's t-test). (G-G') Overlap of btn-lacZ (red) and Klu (green) at 18 h AEL. Klu staining is not detected in the DNH. (H-J) Expression of FMRFa at 18 h AEL in control (H) and klu mutants expressing gbb directly in the DNH (I) and klu mutants expressing gbb directly in the Ap4 neuron itself (J). There is no rescue of FMRFa expression in these genetic backgrounds. The percentage of FMRFa-expressing cells is indicated bottom right. (K) Percentage FMRFa-expressing cells in the various genotypes. n≥10 VNCs. Wild-type and mutant VNCs were stained and analyzed on the same slide. (A) ap-Gal4/UAS-nmEGFP; (B) ap-Gal4/UAS-nmEGFP, klu<sup>1208STC</sup>/klu<sup>1208STC</sup>; (C) ap-Gal4/UAS-nmEGFP>btn-lacZ; (D) ap-Gal4,btn-lacZ; klu<sup>1208STC</sup>/klu<sup>1208STC</sup>; (E) btn-lacZ; (G) OregonR; (H) slit-Ga4, UAS-gbb; klu<sup>1208STC</sup>/klu<sup>1208STC</sup>; (I) ap-Gal4, UAS-gbb; klu<sup>1208STC</sup>/klu<sup>1208STC</sup>.
specification of the DNH itself, which could result in the absence of Gbb in this gland. Although we did not observe Klu expression in the DNH (Fig. 4G), it is possible that klu plays an early role in DNH specification. To circumvent this issue, we attempted to rescue FMRFa in klu mutants by providing gbb directly to the DNH (slit-Gal4/UAS-gbb in the klu mutant). However, this failed to restore FMRFa expression (Fig. 4I,K). Hence, these results lead to the conclusion that the Ap4 neuron is unable to respond to Gbb. To test this, we provided gbb in the Ap4 neuron itself using ap-Gal4/UAS-gbb in klu mutants [previous studies reported that the misexpression of gbb rescues gbb mutants cell-autonomously (Allan et al., 2003)]. However, we found no rescue of FMRFa in this genetic background (Fig. 4I,K). Thus, the Ap4 neuron is not competent to respond to the Gbb ligand in klu mutants.

Expression of type I BMP receptors rescues FMRFa expression in the Ap4/FMRFa neuron

In the Ap4/FMRFa cell, activation of the BMP pathway begins when the ligand Gbb binds to a tetrameric membrane receptor complex that consists of two receptor pairs—the type I and type II BMP receptors (Allan et al., 2003). Then, the constitutively active type II BMP receptors [wishful thinking (wit)] recruit and then phosphorylate their type I BMP partners [saxophone (sax) and thickveins (tkv)]. The type I receptors in turn phosphorylate the cytoplasmic receptor-regulated Smad (R-Smad) Mad. The R-Smad Mad then associates with common non-phosphorylated Smads (co-Smads) (Medea in Drosophila) to form a phospho-Mad complex (pMad) that translocates to the nucleus to participate in transcriptional regulation (Fig. 5A) (reviewed by Keshishian and Kim, 2004).

Why then is the Ap4 neuron unable to respond to the Gbb ligand in klu mutants? To address this issue, we examined whether constitutive activation of the BMP pathway is able to rescue FMRFa expression in klu mutants. In Drosophila, constitutive activation of the BMP pathway can be achieved by expression of activated versions of either one of the type I receptors sax or tkv [UAS-saxA and UAS-tkvA (Haerry et al., 1998)]. Is the klu mutant Ap4 neuron able to transduce the signal from activated type I BMP receptors? Using elav-Gal4, we expressed these modified receptors and found that this resulted in a 68% rescue of FMRFa (Fig. 5B,D). We also observed ectopic FMRFa-expressing cells in the Ap cluster. These results indicate that klu mutant Ap4/FMRFa neurons are defective in their response to the Gbb ligand.

To initiate downstream responses, the type I BMP receptors need to be recruited and phosphorylated by the type II receptor Wit, and this phosphorylation step is activated by binding of the BMP ligand to the type I BMP receptors in the receptor complex. Therefore, expression of constitutively active forms of the type I BMP receptors Sax and Tkv bypasses the need for the type II receptor Wit, whereas the expression of normal forms of these receptors does not. To determine whether the klu phenotype is due to type I or type II BMP receptors, we first tested the involvement of the type II receptor wit by expressing the wild-type versions of the type I BMP receptors (elav>sax,tkv in the klu mutant). Even in this scenario, we found a similar rescue of FMRFa expression in klu mutants (66%; Fig. 5C,D), demonstrating that type I and not type II receptors are at the core of the klu phenotype of the Ap/FMRFa neuron. To test this notion further, we attempted to rescue FMRFa expression in klu mutants by expressing the type II receptor Wit (elav>G4-UAS-wit in the klu mutant). However, we found no statistically significant rescue in this genetic background (Fig. 5D). Thus, our results indicate that klu controls, directly or indirectly, the levels or responsiveness of the plasma membrane-bound type I BMP receptors in the Ap4/FMRFa neuron.

None of the known Ap neuron determinants controls Klu expression

Next, we addressed the activation of klu at the very end of the NBS-6T lineage, when the Ap4/FMRFa neuron is generated. Given that we cannot rule out the possibility that the Ap4 neuron expresses Klu due to inheritance from the neuroblast, we examined Klu expression both within the neuroblast at stage late 14 (when the neuroblast is generating the Ap4 cell) and within the whole Ap cluster. In those mutants in which the Ap and Eya markers are lost, we used the lher (K) reporter, and we identified the Ap cluster by position. Mutants for the cas, nab and sqz temporal/subtemporal genes had no apparent change in Klu expression (supplementary material Fig. S2A,B,F-I,Q-S). grh and svp mutants displayed an increase in the number of Ap neurons (Baumgardt et al., 2009; Benito-Sipos et al., 2011) and, accordingly, there was an increase in Ap neurons expressing Klu at stage 16 (supplementary material Fig. S2D,K,Q-S). However, Klu expression was turned off normally at 18 hAEL in both mutants (supplementary material Fig. S2E,L,Q-S). We concluded that the increase in Klu-positive neurons displayed by grh and svp embryos merely reflects the increase in the number of Ap neurons.
Finally, we studied Klu expression in mutants for the col, ap and eyu determinants, but observed no apparent global effect upon Klu expression in these mutant backgrounds (supplementary material Fig. S2M-S). Therefore, none of the reported Ap neuron determinants controls Klu expression, and the factor(s) involved in the Klu activation remain(s) elusive.

**DISCUSSION**

We find that Klu is expressed in the newly born Ap4/FMRFa neuron and that this expression is maintained until the end of stage 17. Our results demonstrate that the key role of Klu in this scenario is in the control of type I BMP receptor expression. Nevertheless, our findings indicate a pleiotropic function of Klu in Ap cluster specification in general and particularly in Ap4 neuron differentiation, indicating that Klu is a multitasking transcription factor. Finally, we found that transient suppression of klu is crucial for specification of the Ap4/FMRFa neuron. These results unravel a new and non-canonical role of Klu in neural cell specification.

**A novel role of Klu in neural cell specification**

Previous studies carried out in the NB4-2 lineage reported that Klu prevents the second-born daughter cell in that lineage (GMC4-2b) from adopting the GMC4-2a fate (Yang et al., 1997). This is achieved by repressing eve expression in GMC4-2b, and loss of klu expression leads to a duplication of GMC4-2a fate. However, owing to the limited availability of lineage markers, the role of Klu in both later born GMCs in the NB4-2 lineage and other NB lineages has not been examined (Klein and Campos-Ortega, 1997; McDonald et al., 2003; Yang et al., 1997). Recent studies pinpoint the transcription factor Klu as distinguishing a type II neuroblast from an INP in larval brain. Klu functions to maintain the identity of type II neuroblasts, and klu mutant larval brains show progressive loss of type II neuroblasts due to premature differentiation (Xiao et al., 2012). Additionally, studies from sensory organ precursors (SOPs) suggest a similar mechanism of action: overexpression of Klu results in the formation of supernumerary bristles, whereas loss-of-function leads to loss of bristles due to the lack of determination of the corresponding SOP (Kaspar et al., 2008). Hence, all previous data concerning Klu indicate that it distinguishes between two fates (‘A’ versus ‘B’). In all these cases, loss-of-function of klu produces extra cells with identity ‘A’, whereas gain-of-function of klu produces extra cells with identity ‘B’.

However, our studies of NB5-6 reveal a different role for Klu. Loss- and gain-of-function of klu do not permit identity within the lineage (as the canonical role of klu would predict). On the contrary, Klu is necessary for the proper initiation of one of the components of the combinatorial code necessary for Ap4/FMRFa specification: BMP signaling. Importantly, neither BMP signaling nor Klu is sufficient to activate FMRFa expression in other Ap neurons. Therefore, our results highlight a novel function of the transcription factor Klu in neural cell specification.

**Klu, like its mammalian ortholog WT1, has self-renewal or differentiation functions during development depending on context**

klu encodes a transcriptional regulator characterized by four zinc-finger motifs in the C-terminus and is the fly ortholog of mammalian WT1 (Klein and Campos-Ortega, 1997; Yang et al., 1997). Inconsistent and contradictory functions have been ascribed to WT1, which can act as a transcriptional activator or repressor, promoting proliferation, differentiation or apoptosis, in a highly context-dependent manner. In mammals, mutations in WT1 result in tumor formation, and WT1 has also been found necessary for the proliferation of certain neuronal progenitors (reviewed by Hohenstein and Hastie, 2006; Roberts, 2005), which is in line with the reported role of Klu in self-renewal. By contrast, WT1 has also been identified in differentiation process, such as playing an essential role in nephron progenitor differentiation during renal development (reviewed by Hohenstein and Hastie, 2006; Roberts, 2005) and participating in the differentiation of the olfactory epithelium (Wagner et al., 2005), in agreement with the role of Klu reported here. Therefore, our findings indicate that Klu, like WT1, has either self-renewal or differentiation functions during development depending on context.

**Klu is required for the onset, but not for the maintenance, of BMP signaling within the Ap4 neuron**

We have undertaken a number of experiments involving different markers, mutant combinations and a detailed analysis of Klu with respect to the FMRFa phenotype within the Ap cluster. Our findings reveal that at the heart of the klu phenotype in the Ap4/FMRFa neuron lay defects in BMP signaling. Previous studies reported that FMRFa expression is maintained by persistent retrograde BMP signaling in the Ap4 neuron (Eade and Allan, 2009). Here, we find that klu is a crucial regulator of BMP signaling. However, Klu expression is extinguished at the end of stage 17. Hence, Klu is necessary for the initiation of BMP signaling but is dispensable for its maintenance within the Ap4 neuron. Further investigation will be necessary to elucidate the molecular function of klu in controlling the expression of type I BMP receptors.

Is the control of BMP signaling a global/conserved function of Klu? klu mutants do not display a general absence of pMad staining within the whole VNC (data not shown). Hence, although we cannot rule out global control of type I BMP receptors by klu, its role does seem to be highly context dependent. Additionally, previous studies by ChIP-chip in embryonic mouse kidney tissue identified transcriptional targets of WT1 in nephron progenitor cells during renal development in vivo. Among these targets they found several components of the BMP signaling pathway: Bmp4, Bmp7, the two R-Smads Smad3 and Smad4, and the two inhibitory Smads (I-Smads) Smad6 and Smad7. However, none of the BMP receptors was found. Hence, although WT1 has been associated with the control of BMP signaling, the mechanisms underlying this control seem to be multiple and highly cell specific. Intriguingly, in those studies, Nab1 and Nab2, the mammalian orthologs of the subtemporal factor Nab, were also found to be targets of WT1. Here, we found in Drosophila that Nab expression was unaffected by loss-of-function of klu.

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**Competing interests statement**

The authors declare no competing financial interests.

**Supplementary material**

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