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Jeb/Alk signalling regulates the Lame duck GLI family transcription factor in the *Drosophila* visceral mesoderm

Dmitry Popichenko, Fredrik Hugosson, Camilla Sjögren, Murat Dogru, Yasuo Yamazaki, Georg Wolfstetter, Christina Schönherr, Mahsa Fallah, Bengt Hallberg, Hanh Nguyen* and Ruth H. Palmer‡

SUMMARY

The Jelly belly (Jeb)/Anaplastic Lymphoma Kinase (Alk) signalling pathway regulates myoblast fusion in the circular visceral mesoderm (VM) of *Drosophila* embryos via specification of founder cells. However, only a limited number of target molecules for this pathway are described. We have investigated the role of the Lame Duck (Lmd) transcription factor in VM development in relationship to Jeb/Alk signal transduction. We show that Alk signalling negatively regulates Lmd activity post-transcriptionally through the MEK/MAPK (ERK) cascade resulting in a relocalisation of Lmd protein from the nucleus to cytoplasm. It has previously been shown that downregulation of Lmd protein is necessary for the correct specification of founder cells. In the visceral mesoderm of *lmd* mutant embryos, fusion-competent myoblasts seem to be converted to 'founder-like' cells that are still able to build a gut musculature even in the absence of fusion. The ability of Alk signalling to downregulate Lmd protein requires the N-terminal 140 amino acids, as a $Lmd^{141\text{-}866}$ mutant remains nuclear in the presence of active ALK and is able to drive robust expression of the Lmd downstream target Vrp1 in the developing VM. Our results suggest that Lmd is a target of Jeb/Alk signalling in the VM of *Drosophila* embryos.

KEY WORDS: Lmd, Alk, Jeb, *Drosophila*, Visceral muscle, Founder cell, Fusion competent myoblast

INTRODUCTION

Mammalian GLI proteins, together with the GLIS (GLI-similar) and ZIC (zinc-finger protein of the cerebellum) proteins are Krüppel-like zinc-finger proteins that act as both activators and repressors of transcription (Kang et al., 2010). Although GLI proteins are well studied, the physiological functions of the related GLIS and ZIC proteins are less well understood. In the fruitfly, the homologues of GLI, ZIC and GLIS are encoded by the *cubitus interuptus* (*ci*), *odd-paired* (*opa*) and *lame duck* (*lmd*)/*myoblasts incompetent* (*minc*)/*gleeful* (*gfl*) genes. The *Drosophila* Lame Duck (Lmd/Minc/Gfl) zinc-finger transcription factor plays a crucial role in the specification of somatic fusion competent myoblasts in embryos (Duan et al., 2001; Furlong et al., 2001; Ruiz-Gómez et al., 2002). During myogenesis, two cell types – founder cells (FCs) and fusion competent myoblasts (FCMs) – undergo a fusion-process to form syncytial myotubes. In *lmd* mutant embryos, FCM precursors are unable to downregulate *twist* expression and switch on FCM-specific genes, such as *sns*, *hairy* and the myogenic specific transcription factor *mef2* (Duan et al., 2001; Ruiz-Gómez et al., 2002). In the somatic mesoderm of *lmd* mutant embryos, myoblast fusion fails to occur due to the absence of mature FCMs (Duan et al., 2001). Examination of the undifferentiated somatic FCM precursors in *lmd* mutant embryos reveals a fate change that resembles pericardial cells of the dorsal vessel or APM (adult muscle precursor)-like cells (Sellin et al., 2009).

Studies of Lmd transcriptional activity suggest a number of shared transcription factor-binding regions with Mef2 and a function as a co-regulator of Mef2, both as an activator or repressor of myogenic specific genes (Cunha et al., 2010). Modulation of Lmd activity occurs at the post-transcriptional level, in part by regulation of its subcellular localisation (Duan and Nguyen, 2006). Recent analysis has identified genomic regions bound by Lmd that are also co-occupied by key regulators such as Twist, Tinman and Mef2 combinatorially regulating the expression of FCM- and FC-specific genes in the embryonic mesoderm (Busser et al., 2012). Interestingly, recent work indicates a role for the E3 ubiquitin ligase Mind bomb 2 (Mib2) in the regulation of Lmd protein levels (Carrasco-Rando and Ruiz-Gómez, 2008).

In contrast to its exclusive expression in FCMs within the developing somatic muscle, *lmd* mRNA is observed in both FCs and FCMs of the visceral mesoderm (Ruiz-Gómez et al., 2002). We became interested in the function of Lmd in the developing visceral muscle, and the impact of Alk RTK (receptor tyrosine kinase) signalling on Lmd function in the *Drosophila* VM. Alk and its ligand Jeb play a crucial role in midgut development *in vivo*, driving a MAPK (ERK)-mediated pathway required for visceral musculature development (Englund et al., 2003; Lee et al., 2003; Lorén et al., 2003; Lorén et al., 2001; Stute et al., 2004; Weiss et al., 2001). Here, we investigate the role of Lmd in visceral muscle and show that, as in the developing somatic muscle, Lmd protein is specific for the FCM population in the VM. In *alk* mutants, Lmd protein is observed in all cells of the developing VM, whereas activation of Alk signalling by overexpression of Jeb leads to a complete loss of Lmd protein, but not of *lmd* transcripts, in the VM. Thus, Jeb/Alk signalling results in the downregulation of Lmd protein at the post-translational level. In parallel cell culture experiments, both *Drosophila* and human Alk signalling lead to the translocation of Lmd protein from the nucleus to the cytoplasm, where it is then accessible for degradation. In keeping with this hypothesis, activation of Alk signalling in a Mib2 mutant background significantly delays the degradation of Lmd protein in the developing embryonic VM. Examination of Lmd mutants suggests that Alk regulation occurs through the N-terminal 140

Department of Molecular Biology, Building 6L, Umeå University, Umeå S-90187, Sweden.

*Present address: Department of Biology and Division of Developmental Biology, University of Erlangen-Nürnberg, Staudtstrasse 5, Bau A02, 91058 Erlangen, Germany
‡Author for correspondence (ruth.palmer@ucmp.umu.se)

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residues of Lmd, as Alk signalling cannot downregulate the Lmd$^{141-866}$ mutant protein, either in the *Drosophila* embryo or in cell culture. Taken together, these data suggest that, upon visceral muscle fusion, Alk signalling results in redistribution of Lmd protein to the cytoplasm, where it is degraded, thus terminating its activity.

MATERIALS AND METHODS

Fly stocks

Standard protocols were used for *Drosophila* husbandry. *white*^{1118} or blue-balanced sibling embryos are referred to as 'wild-type' controls. Mutant embryos were distinguished by presence of *lacZ*-balancer. *lmd*^{A388}/*minc*^{A388} was from M. Ruiz-Gomez (Ruiz-Gómez et al., 2002). Other strains used were *bagpipe-GAL4* (Zaffran et al., 2001), *UAS-jeb* (Varshney and Palmer, 2006), *handC-GFP* (Sellin et al., 2006), *UAS-lmd*$^{wild\text{-}type/141\text{-}866/PXXP}$ (Duan and Nguyen, 2006; Duan et al., 2001), *alk*^{10} (Lorén et al., 2003), *sns*$^{ZF1.4}$ (Bour et al., 2000), *UAS-mib2* and *UAS-mib2*^{ARF} (Nguyen et al., 2007), *HLH54F-lacZ* (Ismat et al., 2010), *twist2xPE-Gal4* (Bloomington-25707), *Df(2L)Exel8039* uncovering *mib2* (Bloomington-7846) and *Df(3R)BSC527* uncovering *lmd/minc* (Bloomington-25055).

In situ hybridisation

For *in situ* hybridisation of *Drosophila* embryos with DIG-labelled RNA probes to *lmd* and *duf/kirre* (Roche, Basel) we adapted the Berkeley Drosophila Genome Project (BDGP) protocol (Weiszmann et al., 2009).

Immunostaining and antibodies

Embryos were fixed in 4% PFA (dpERK, 8% PFA) and stained as described previously (Lorén et al., 2001). Primary antibodies used were: rabbit anti-β-galactosidase (1:200, Cappel), rabbit anti-phospho-HistoneH3 (1:500, Millipore), mouse anti-β-galactosidase (1:1000, Promega), rabbit anti-Alk (1:1000) (Lorén et al., 2001), guinea-pig anti-Alk (1:1000) (Englund et al., 2003), rabbit anti-Lmd (1:1000) (Duan et al., 2001), mouse anti-dpERK (1:500, Sigma), guinea-pig anti-Jeb (1:1000) (Englund et al., 2003), rabbit anti-Mef2 (1:500), guinea-pig anti-Vrp1 (1:1000) (Eriksson et al., 2010), rabbit anti-β3-tubulin (1:2000) (Leiss et al., 1988), guinea-pig anti-β3-tubulin (1:3000), rat anti-Org-1 (1:100) (Schaub et al., 2012), guinea-pig anti-Mib2 (1:1000) (Carrasco-Rando and Ruiz-Gómez, 2008), mouse anti-fasciculin III (mAb-7G10, 1:50; Developmental Studies Hybridoma Bank) and mouse anti-HA (mAb-16B12, 1:500; Covance). Secondary antibodies coupled to Cy2, Cy3 and Cy5 were from Amersham or Jackson. Embryos were mounted in methylsalicylate or Fluoromount G (SouthernBiotech).

Plasmid constructs and transfection

Lmd-GFP was created by cloning either full-length *lmd*, *lmd*$^{141-866}$ *lmd*$^{1-40}$, *lmd*$^{1-140}$ or *lmd*^{PXXP} mutant cDNAs into the CMV;Cterm-GFP vector and verified by sequencing. Jeb-pIREShrGFP and pcDNA3-ALK have been described previously (Yang et al., 2007). Human wild-type hALK and mutated hAlk^{F1174S} in pcDNA3 have been described previously (Martinsson et al., 2011). HEK293 cells were transfected with lipofectamine (Invitrogen) according to manufacturer's protocol. Inhibitors were added to fresh medium immediately after removal of transfection mixture. Transfected cells were cultured for 16-18 hours, prior to fixation in 4% PFA, and stained with appropriate antibodies. Primary antibodies employed were: rabbit anti-hAlk (1:2000, Abcam) and guinea-pig anti-Alk (1:1000) (Englund et al., 2003). Nuclei were stained with DAPI (0.5 μg/ml). Transfections were performed at least three times independently; and more than 100 GFP and Cy3-positive cells were counted.

Phosphoproteomics analysis

HEK293 cells were transfected with hALK^{F1174S} and Lmd-GFP, and lysed 24 hours post-transfection. Lmd-GFP was immunoprecipitated using GFP-TrapA (ChromoTek) and analysed for phosphorylation sites using mass spectrometry (Keck Proteomics).

Inhibitor treatments

The ALK-specific inhibitor NVP-TAE684 has been described previously (Galkin et al., 2007; Schönherr et al., 2011). HEK293 cells were incubated with 0.2 μM of the ALK inhibitor NVP-TAE684 as indicated. Treatment with 10 μM LY294002 (Calbiochem) or 10 μM U0126 (Calbiochem) was carried out prior to analysis of Lmd protein localisation.

RESULTS

Characterisation of visceral mesoderm development in *lmd* mutants

The Lmd transcription factor is an important early factor in muscle development in the developing *Drosophila* embryo (Duan et al., 2001; Ruiz-Gómez et al., 2002). It has previously been reported that *lmd* mRNA is expressed in both cell types (FCs and FCMs) of the visceral mesoderm (VM) prior to fusion (Ruiz-Gómez et al., 2002). In spite of this, Lmd protein is restricted to FCMs (Fig. 1A). This pattern raises the possibility that the activity of Lmd may be regulated by Alk-mediated signalling in the VM. Examination of Alk signalling in *lmd*$^{-/-}$ embryos suggests that the initial Alk signalling events are not affected, as evidenced by robust MAPK (ERK) activation (Fig. 1D). Given the crucial role for Jeb/Alk signalling in FC specification (Englund et al., 2003; Lee et al., 2003; Stute et al., 2004), we examined Lmd in *alk*$^{-/-}$ embryos, which fail to specify FC fate, observing that both *lmd* mRNA (Stute et al., 2004) and protein are detected in all VM cells (Fig. 1G-I; and data not shown), suggesting that Jeb induced Alk activation may function to downregulate Lmd protein in the VM.

Activation of Alk by the Jeb ligand leads to loss of Lmd protein in the VM

To further investigate whether Lmd is a target of Jeb/Alk signalling, we followed Lmd together with other VM-specific markers upon ectopic expression of Jeb with the VM-specific *bagpipe-GAL4* driver (*bapGal4>UAS-jeb*) (Brand and Perrimon,

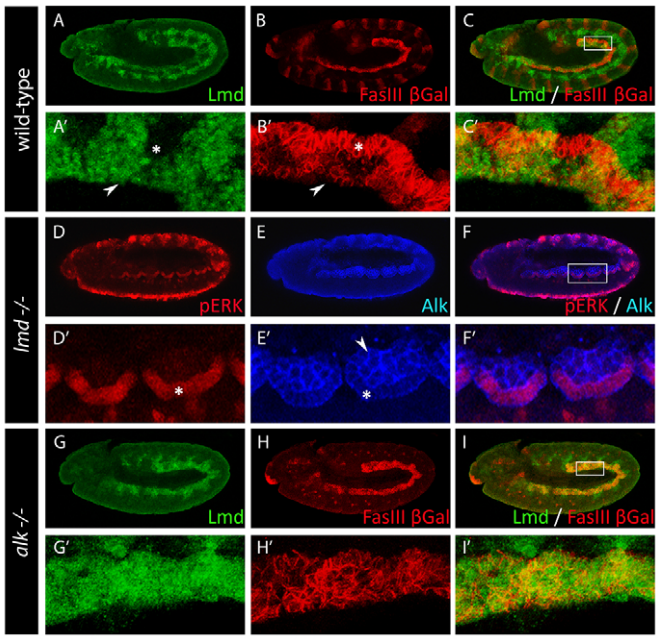

Fig. 1. Lmd expression in the developing embryonic VM. (**A-C′**) In wild-type embryos (stage 11), Lmd protein (green) is found in VM FCMs (arrowheads), but not in columnar FCs (asterisks). (**D-F′**) Alk signalling in FCs of *lmd*$^{-/-}$ mutants is comparable with wild type, as assayed by MAPK (ERK) activation (dpERK in red) in FCs (asterisks). dpERK is not observed in FCMs (arrowhead). (**G-I′**) All VM cells in *alk*$^{-/-}$ embryos (marked with Fas3 in red) express Lmd (green). Boxes in C,F,I mark areas enlarged in C′,F′,I′, respectively. Anti-βGal (in red) identifies controls.

1993), which leads to Alk activation in all VM cells (Englund et al., 2003; Lee et al., 2003; Stute et al., 2004). Under these conditions, we were unable to observe Lmd protein in the VM, although levels were still clearly visible in the nearby somatic mesoderm (Fig. 2A). In wild-type embryos, pERK is seen only in the FC population (in a pattern similar to *lmd*$^{-/-}$, Fig. 1D-F); however, clear phosphorylation of MAPK(ERK) occurs in all VM cells in response to Alk activation, in agreement with previous observations (Englund et al., 2003; Lee et al., 2003; Stute et al., 2004) (Fig. 2A′). The transcription factor Mef2 is a key regulator of muscle differentiation (Bour et al., 1995; Lilly et al., 1995), and is directly regulated by Lmd in somatic mesoderm (Duan et al., 2001). By contrast, Mef2 expression is unaffected in VM of *lmd*$^{-/-}$ (not shown) or *bapGAL4>UAS-jeb* embryos (Fig. 2B). Verprolin (Vrp1/Wip/Sltr) is a cytoskeletal co-regulator of the Arp2/3 complex that is exclusively expressed in FCMs (Eriksson et al., 2010; Kim et al., 2007; Massarwa et al., 2007). In *lmd*$^{-/-}$ mutants, neither *Vrp1* mRNA nor protein are expressed in developing somatic muscle (Kim et al., 2007; Massarwa et al., 2007). In support of a similar role for Lmd in the VM we observe that Vrp1 is completely abolished from VM but not somatic mesoderm in *bapGAL4 >UAS-jeb* embryos (Fig. 2C). The bHLH transcription factor Hand is specifically expressed in FCs of the VM of wild-type embryos (Kölsch and Paululat, 2002; Lo et al., 2007) and is a transcriptional target of Alk signalling (Varshney and Palmer, 2006). In *lmd*$^{-/-}$ mutant embryos, expression of *hand* is expanded to all VM cells (Popichenko et al., 2007). The *handC-GFP* reporter (Sellin et al., 2006) was used to investigate *hand* expression in response to Jeb expression. In *bapGAL4>UAS-jeb* embryos, GFP is detected in all cells of the VM (Fig. 2D). Thus, activation of Alk signalling by ectopic expression of Jeb results in a loss of cells expressing FCM markers in the VM, with a concomitant increase in cells expressing FC markers.

Loss of Lmd protein function leads to inappropriate cell specification in the VM

In *alk*$^{-/-}$ embryos, specification of visceral FCs does not occur, and all VM cells express FCM-specific genes, such as *sns* and *Vrp1* (Eriksson et al., 2010). Conversely, FC markers such as Org-1 (Lee et al., 2003; Schaub et al., 2012) are absent in *alk*$^{-/-}$ mutants (Fig. 3C, compare with 3A; supplementary material Fig. S1). By contrast, in either *lmd*$^{-/-}$ mutants or embryos in which Alk signalling has been ectopically activated (*bapGAL4>UAS-jeb*) all VM cells are positive for FC-specific markers such as Org-1 (Fig. 3B,D; Fig. 5; Fig. 10B; supplementary material Fig. S2), *hand* (Popichenko et al., 2007; Varshney and Palmer, 2006) and *duf/kirre* (Lee et al., 2003; Stute et al., 2004; Varshney and Palmer, 2006), and show reduced expression of FCM-specific genes, such as *sns* (Lee et al., 2003; Ruiz-Gómez et al., 2002; Stute et al., 2004) and *Vrp1* (Fig. 2C; Fig. 3B; supplementary material Fig. S2). Thus, both precise regulation of Alk activity as well as Lmd protein function appear to be necessary for correct specification of FC and FCM populations in the VM.

The visceral musculature is disorganised in *lmd* mutant and Jeb-overexpressing embryos

One hypothesis arising from these observations is that activation of the Jeb/Alk pathway regulates Lmd in the VM. Therefore, we examined both circular and longitudinal visceral muscles in *lmd*$^{-/-}$ and *bapGAL4>UAS-jeb* embryos. As all VM cells in *lmd*$^{-/-}$ and *bapGAL4>UAS-jeb* embryos display FC features, we expected to observe fusion defects in this tissue owing to an absence of FCM, with a subsequent loss of properly formed gut musculature. For analysis of circular gut musculature, we used *handC-GFP*, which is expressed in nuclei of the circular VM (Sellin et al., 2006). In wild type, binuclear myotubes can be observed stretching dorso-ventrally to enclose the gut epithelium (Fig. 4A′), with GFP-positive cell nuclei forming distinguishable rows along the gut (two on each side). This arrangement is visible from stage 14-15 (Fig. 4A) and is clearly obvious by stage 16-17 (Fig. 4D). In the absence of Lmd (*lmd*$^{-/-}$ or

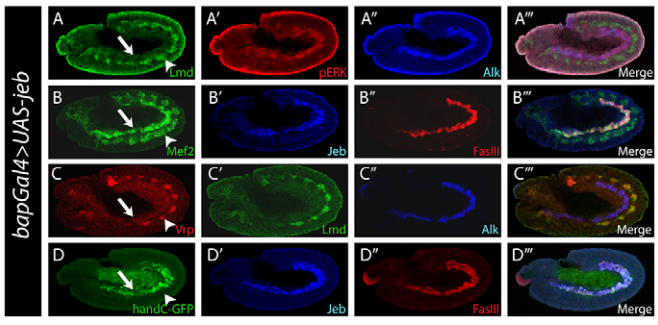

Fig. 2. Characterisation of mesodermal specification in *bapGal4>UAS-jeb* embryos. (**A-A‴**) Lmd protein (green) is detected only in SM (arrowhead) but not VM (arrow) (Alk in blue; dpERK in red) of *bapGal4>UAS-jeb* embryos. (**B-B‴**) Mef2 (green) is expressed in both SM and VM of *bapGal4>UAS-jeb* embryos; Jeb (blue) and Fas3 (red) stain the VM. Arrow and arrowhead indicate VM and SM, respectively. (**C-C‴**) Vrp1 (red) shows a similar pattern of expression to Lmd (green) in SM and is undetectable in VM of *bapGal4>UAS-jeb* embryos (arrow). Alk is in blue. Arrowhead indicates SM. (**D-D‴**) *handC-GFP* (green) is expressed in all VM cells of *bapGal4>UAS-jeb* embryos, which are marked with Jeb (blue) and Fas3 (red). Arrow and arrowhead indicate VM and SM, respectively.

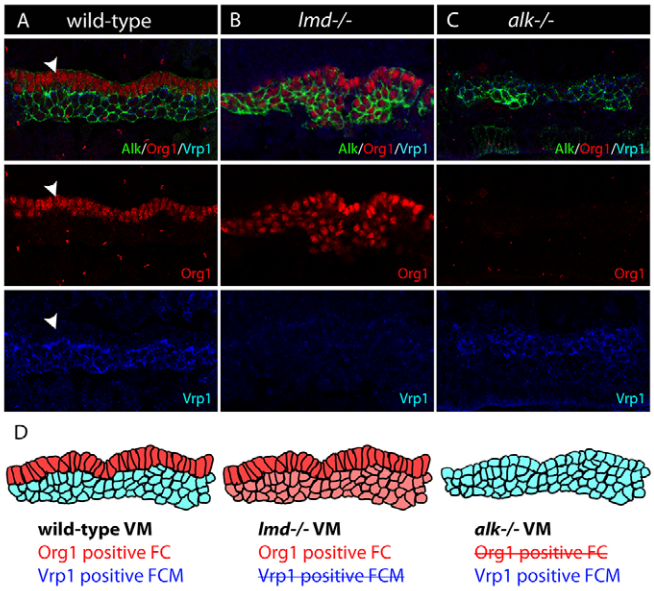

Fig. 3. VM in *lmd* mutants displays FC characteristics. (**A**) In wild-type embryos (stage 11) Org-1 protein (red) is observed in visceral FCs (arrowheads), but not in FCMs. VM cells express Alk (green); FCMs express Vrp1 (blue). (**B**) VM cells in *lmd*$^{-/-}$ embryos (marked with Alk in green) are not Vrp1 (blue) positive, but do express Org-1 (red). (**C**) Org-1 (red) is absent in *alk*$^{-/-}$ embryos, whereas Vrp1 expression is observed (blue). (**D**) VM cell identity in wild-type, *lmd*$^{-/-}$ and *alk*$^{-/-}$ animals; Org-1-positive cells in red; Vrp1-positive cells in blue.

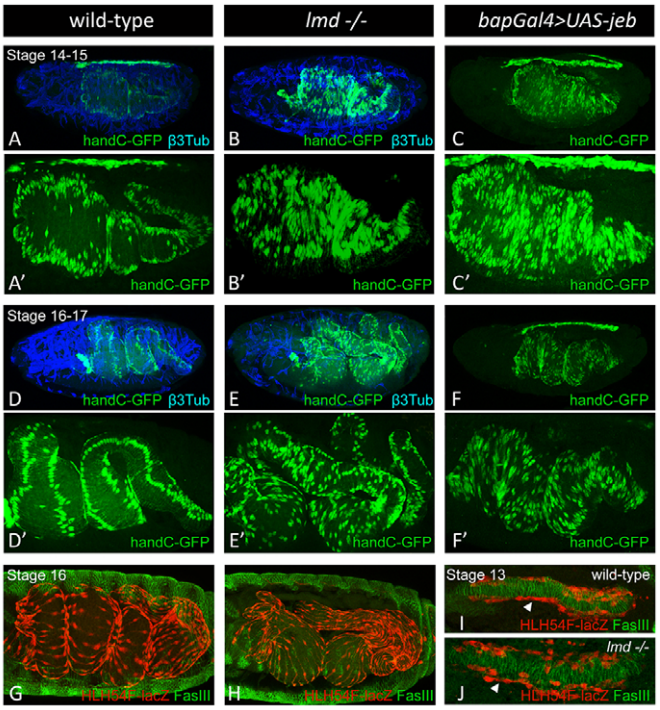

Fig. 4. *lmd*$^{-/-}$ and *bapGal4>UAS-jeb* embryos show similar defects in gut musculature. (**A**,**A′**,**D**,**D′**) GFP-positive nuclei of the circular myotubes (*handC-GFP* in green) in wild-type embryos are located in four rows along gut from stage 14-15 (A) until stage 17 (D). (**B-C′**) In *lmd*$^{-/-}$ (B,B′) and *bapGal4>UAS-jeb* (C,C′) embryos at stage 14-15; *handC-GFP*-positive nuclei are randomly distributed within the visceral muscle. (**E-F′**) At later stages, an irregular sheet of 'myotubes' encloses the gut. (**G**,**H**) Longitudinal myofibres (*HLH54F-lacZ* in red) in wild-type stage 16 embryos are arranged along the gut (G). In stage 16 *minc(lmd)*^{A388}/*Df(3R)BSC527* mutant embryos, aberrant portioning of the gut is observed (H). (**I**,**J**) Stage 13 wild-type (I) developing myofibres migrate and elongate (arrowhead), whereas in *minc(lmd)*^{A388}/*Df(3R)BSC527* mutants (J) longitudinal visceral cells migrate but do not elongate (arrowhead).

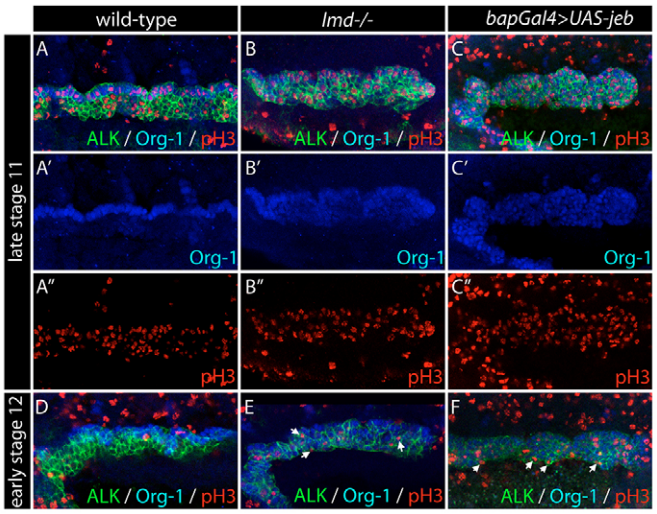

Fig. 5. Increased cell proliferation in the VM of *lmd*$^{-/-}$ and *bapGal4>UAS-jeb* embryos. (A-F) Alk (green), Org-1 (blue) and phospho-Histone-H3 (pH3, red). (**A-C**) Late stage 11, wild-type (A), *lmd/minc*^{A388} (*lmd*$^{-/-}$) (B) and *bapGal4 >UAS-jeb* (C) embryos. (**A′**) FC-specific expression of Org-1 in VM of wild type. (**B′**) Org-1 expression expands to all VM cells in *lmd*$^{-/-}$ mutants. (**C′**) *bapGal4>UAS-jeb* embryos exhibit robust expression of Org-1 within all VM cells. (**A″**) Anti-pH3-positive nuclei are detectable in nearly all visceral FCs but also in some FCMs in wild type. (**B″**,**C″**) pH3-positive visceral myoblasts in *lmd*$^{-/-}$ mutant and *bapGal4>UAS-jeb* embryos. (**D-F**) At early stage 12, *lmd/minc*^{A388} (*lmd*$^{-/-}$) (E) and *bapGAL4 >UAS-jeb* (F) embryos display increased numbers of dividing cells (arrows), in contrast to the wild-type situation (D).

bapGAL4>UAS-jeb), GFP-positive nuclei appear to be increased in number and no longer form four organised rows, but are randomly distributed at stage 14-15 (Fig. 4B,C). These cells are still able to envelope the gut completely forming an irregular sheet by stage 16-17 (Fig. 4E,F). FCMs of trunk VM are also a FCM source for the multinuclear longitudinal gut muscles, leading us to investigate their appearance in *lmd*$^{-/-}$ mutants. At stage 13, *HLH54F-lacZ* expression is detected in developing longitudinal visceral myotubes that migrate along the circular gut musculature and elongate upon fusion (Fig. 4I). By later stages, 18-22 rows of longitudinal muscles arrange as an outer muscle layer around the mature gut (Fig. 4G) (Klapper, 2000). In *lmd*$^{-/-}$ embryos, migration of the longitudinal muscles appear normal and cells adopt a spindle-like shape by forming cellular protrusions (Fig. 4J). However, *HLH54F-lacZ*-positive cells retain this shape during the migration process and can therefore be distinguished from the multinucleated, flat-shaped longitudinal myotubes of wild-type embryos (compare arrowheads in Fig. 4I,J). At the end of embryogenesis, we observed an aberrant portioning of the overall gut structure in *lmd*$^{-/-}$ mutants and, in contrast to the circular visceral muscle development, distances between single longitudinal nuclei appear to be increased in mutants (Fig. 4H).

Given the observed gene expression profiles and the visceral phenotype in *lmd*$^{-/-}$ embryos, we conclude that in the absence of Lmd protein all cells of trunk VM acquire a more FC-like cell identity. Interestingly, although there are no FCMs to fuse with, these cells are still able to form a circular gut musculature, albeit a disorganised one. Similar phenotypes are observed in both *lmd*$^{-/-}$ and *bapGAL4>UAS-jeb* animals.

As *lmd*$^{-/-}$ mutant and *bapGAL4>UAS-jeb* embryos appear to have increased numbers of *handC-GFP*-positive nuclei, we further investigated them with the cell division marker phospho-HistoneH3 (pH3). During stage 11, pH3-positive nuclei within the VM are visible in both FCMs and FCs (Fig. 5A-A″). VM of *lmd*$^{-/-}$ mutant and *bapGAL4>UAS-jeb* embryos exhibit a slight overall increase of pH3-positive nuclei (Fig. 5B-C″). By stage 12, the number of dividing cells is dramatically reduced in wild type (Fig. 5D), in striking contrast with *lmd*$^{-/-}$ mutant and *bapGAL4>UAS-jeb* embryos where persistent mitosis is observed (Fig. 5E,F, arrows). Thus, in addition to transformation of visceral FCMs into a FC-like state, persistent mitosis may also contribute to the visceral muscle phenotype observed.

Alk signalling regulates Lmd protein post-transcriptionally

The mechanism underlying the regulation of Lmd by Alk signalling is unknown. Although Lmd protein is clearly downregulated by ectopic Alk signalling, *lmd* mRNA is still expressed in VM of *bapGAL4>UAS-jeb* embryos (Fig. 6D). This mirrors the wild-type situation, where we observe a lack of Lmd protein in FCs, in a background of *lmd* mRNA expression. This suggests that downregulation of Lmd by activation of Jeb/Alk signalling occurs post-transcriptionally.

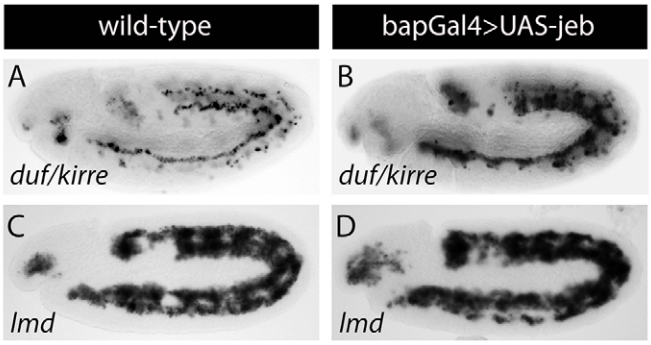

Fig. 6. *lmd* mRNA is expressed in the VM of both wild-type and *bapGal>UAS-jeb*. (**A**,**B**) *Duf/kirre* mRNA detection was employed as a FC-specific control. Upon Jeb overexpression (*bapGal4>UAS-jeb*), all VM cells express *duf/kirre*. (**C**,**D**) *lmd* mRNA is expressed in VM of both wild-type and *bapGal4>UAS-jeb* embryos at stage 11. Lateral views are shown.

Previously, it has been shown that transcriptional activity of Lmd protein depends on its subcellular localisation (Duan and Nguyen, 2006), prompting us to consider the effect of Alk signalling on Lmd subcellular localisation. HEK293 cells were employed to investigate Lmd localisation in response to Alk activation. Lmd-GFP protein accumulated in the nucleus of cells transfected with mock vector, or with inactive Alk (Fig. 7A). By contrast, addition of Jeb-containing conditioned medium (Yang et al., 2007) results in the relocalisation of Lmd-GFP from the nucleus to the cytoplasm (Fig. 7B,H). Given this robust effect of Lmd relocalisation in response to Alk activation, we tested whether this was also the case with gain-of-function human *ALK* mutations, which occur in neuroblastoma (Carén et al., 2008; Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Mossé et al., 2008). In these experiments, we employed gain-of-function human ALK^{F1174S}, which is a ligand-independent mutant receptor observed in neuroblastoma (Martinsson et al., 2011). HEK293 cells transfected with both Lmd-GFP and human ALK^{F1174S} resulted in exclusive localisation of Lmd-GFP in the cytoplasm (Fig. 7C,H). Addition of an ALK-specific inhibitor, NVP-TAE684 (Galkin et al., 2007) results in nuclear retention of Lmd-GFP, suggesting that the nuclear exclusion observed in the presence of human ALK^{F1174S} specifically requires ALK kinase activity (Fig. 7D,H). Thus, we conclude that Lmd-GFP undergoes nuclear-cytoplasmic translocation upon activation of Alk signalling. In mammals, Alk signalling upregulates several pathways: such as Ras/MAPK, PI3K/pAkt, Jak/STAT, PLCγ and C3G/Rap1, whereas in the *Drosophila* VM only the Ras/MAPK pathway has been confirmed *in vivo* (Englund et al., 2003; Lee et al., 2003; Stute et al., 2004). To further define which of these pathways may be important downstream of Alk for Lmd-GFP cellular localisation, we used specific inhibitors of these pathways. Addition of LY294002, an inhibitor of PI3K/pAkt signalling, did not affect cytoplasmic localisation of Lmd-GFP in the presence of human ALK^{F1174S}. However, addition of the MEK inhibitor U0126 was efficient in blocking ALK-driven Lmd-GFP relocalisation, resulting in a significant proportion of cells positive for nuclear Lmd-GFP (Fig. 7F,H).

Alk activity results in the nuclear to cytoplasmic relocalisation of Lmd protein

Alk signalling may function by blocking Lmd translocation to the nucleus or by actively relocalising Lmd from the nucleus to the

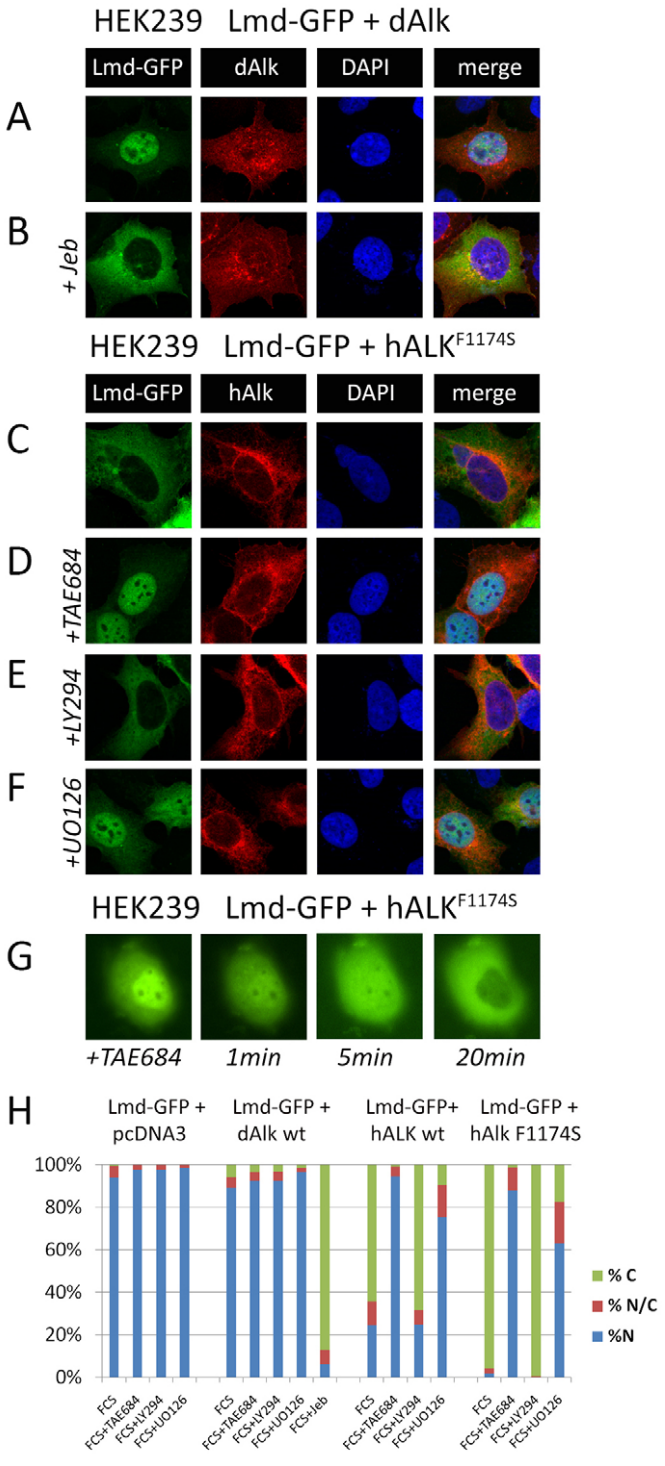

Fig. 7. Alk signalling regulates the subcellular localisation of Lmd. (**A**,**B**) Lmd-GFP (green) is localised exclusively in the nucleus (DAPI, blue) in presence of inactive Alk (red) (A), whereas activation of Alk signalling by Jeb leads to translocation of Lmd-GFP to the cytoplasm (B). (**C-F**) Subcellular localisation of Lmd-GFP in presence of a constitutively active hALK^{F1174S} mutant alone (C), with the ALK inhibitor NVP-TAE684 (D), the PI3K inhibitor LY294002 (E) and the MEK inhibitor U0126 (F). (**G**) Snapshots of live-imaging analysis of cells co-transfected with Lmd-GFP and hALK^{F1174S} in presence of NVP-TAE684. Wash-out of NVP-TAE684 results in Lmd-GFP relocation from the nucleus to cytoplasm within 20 minutes. (**H**) Quantification of results (C, cytoplasmic; N, nuclear).

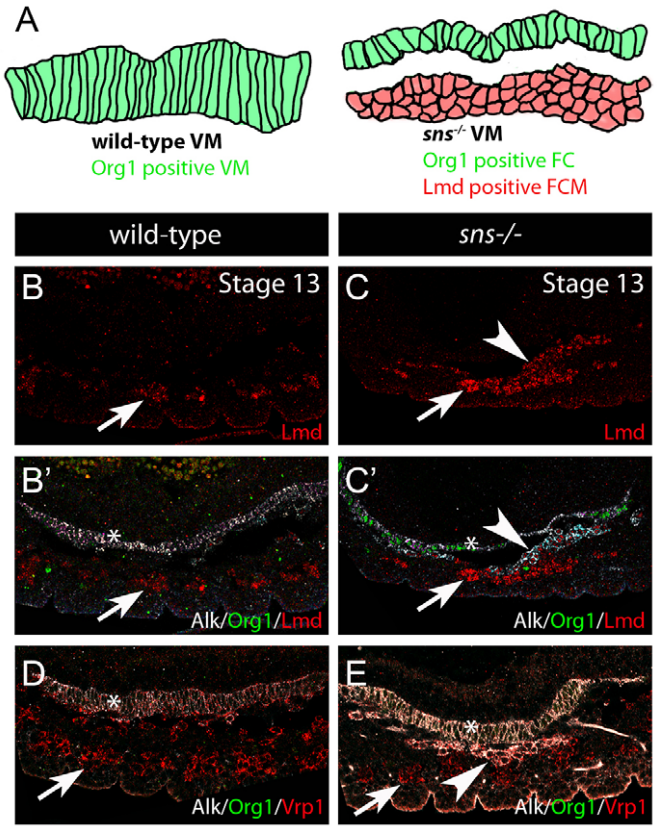

Fig. 8. Lmd protein persists in the VM of *sns* mutants. (**A**) Schematic summary of VM cell identity in wild-type and *sns*$^{-/-}$ animals, indicating Org-1- (green) and Lmd- (red) positive cells in red. In wild-type stage 13 embryos, VM cells are Org-1 positive and Lmd negative upon fusion (left panel). In stage 13 *sns*$^{-/-}$ embryos, fusion is blocked, leading to separation of FC and FCM populations. Lmd can still be detected in visceral FCMs at later stages (right panel). (**B**,**B′**) VM cells in wild-type embryos (Alk in white, asterisk) do not exhibit detectable Lmd (red) at stage 13. Arrows indicate Lmd expression in somatic FCM cells. (**C**,**C′**) Lmd protein (red, arrowheads) expression in visceral FCMs persists in *sns*$^{-/-}$ embryos, and is not detectable in Org-1-positive (green) FCs (asterisk). FC and FCM populations are marked with anti-Alk (white). Arrows indicate Lmd expression in somatic FCMs. (**D**,**E**) VM cells in wild-type (D) or *sns*$^{-/-}$ (E) embryos (Alk in white, asterisk) express high Vrp1 levels (red, arrowhead). Arrows indicate Vrp1 expression in somatic FCM cells.

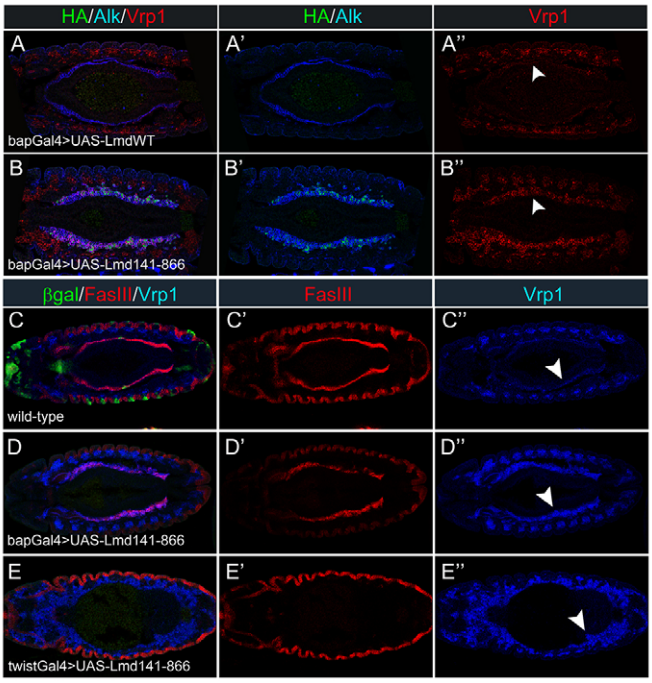

Fig. 9. Expression of Lmd$^{141-866}$ in the VM drives robust expression of Vrp1. (**A-A″**) In *bapGal4>UAS-lmd* embryos (stage 13), Vrp1 protein (red) is downregulated in the VM (Alk in blue) after fusion (A″; arrowhead). (**B-B″**) VM cells in *bapGal4>UAS-lmd*$^{141-866}$-expressing embryos (Alk in blue) express high levels of Vrp1 (red; arrowhead in B″), which is not downregulated. The Lmd$^{141-866}$ protein, in contrast to ectopically expressed wild-type Lmd, is still detectable (B,B′, green). (**C-C″**) In wild type (stage 13), Vrp1 protein (blue) is observed in visceral FCMs (marked with Fas3 in red, C′) and is downregulated after fusion (C″; arrowhead). (**D-D″**) All VM cells in *bapGal4>UAS-lmd*$^{141-866}$-expressing embryos (marked with Fas3 in red, D′) display high levels of Vrp1 (blue; arrowhead in D″). (**E-E″**) In *twistGal4>UAS-lmd*$^{141-866}$ both VM and SM express high levels of the Lmd target Vrp1 (blue; arrowhead in E″). No Fas3-positive VM can be observed (red in E′).

cytoplasm, or both. To better understand the modulation of Lmd localisation by Alk, we performed live cell imaging (Fig. 7G). Wash-out experiments removing NVP-TAE684 from the medium were carried out. Within 20 minutes of NVP-TAE684 removal, all previously nuclear Lmd-GFP signal was detected in the cytoplasm (Fig. 7G), supporting a role for Alk activity in the relocalisation of Lmd from the nucleus. By contrast, incubation of cells with freshly added NVP-TAE684 for at least for 2 hours did not affect Lmd-GFP localisation (data not shown). In addition, removal of NVP-TAE684 was examined in the presence of cycloheximide to block *de novo* protein synthesis (supplementary material Fig. S3). Cycloheximide addition did not significantly affect the cytoplasmic localisation of Lmd-GFP protein, suggesting that *de novo* Lmd-GFP protein synthesis of Lmd-GFP does not play a contributing factor. Taken together, our results suggest a mechanism of action in which activation of Alk signalling through the Ras/MAPK pathway results in subcellular relocalisation of Lmd protein from the nucleus to the cytoplasm.

Lmd protein persists in FCMs of the *sns* fusion mutant

Myoblast fusion in the VM leads to the exposure of FCMs to FC-expressed proteins and vice versa. One hypothesis is that cell fusion might transfer Alk activity into Lmd-expressing FCMs, with the ultimate effect being Lmd downregulation. This hypothesis can be tested in *sns* mutants, in which adhesion between FCs and FCMs is defective and, as a result, myoblasts fail to fuse (Bour et al., 2000). Thus, in such mutants, Lmd protein is not exposed to the FC-specific Alk activity. Indeed, investigation of Lmd protein in *sns*$^{-/-}$ mutants supports this mechanism of Lmd downregulation (Fig. 8), as expression of Lmd protein can still be detected in visceral FCMs at later stages (Fig. 8C). This is in contrast with wild-type embryos, in which Lmd protein is no longer detectable at this stage (Fig. 8B).

The N-terminal is required for regulation of Lmd *in vivo* and *in vitro*

In keeping with our data, an earlier study showed that post-transcriptional mechanisms regulate Lmd activity (Duan and Nguyen, 2006). We employed a number of Lmd mutant transgenes

and examined the effects of overexpressing them in the visceral muscle with *bapGAL4*. Only one Lmd mutant, Lmd$^{141\text{-}866}$, which has a deletion of the N-terminal 140 residues generated a phenotype when ectopically expressed in the VM. *bapGAL4>UAS-Lmd$^{141\text{-}866}$* animals die as late embryos/first instar larvae. Indeed, expression of *UAS-Lmd$^{141\text{-}866}$*, in contrast to wild-type *UAS-Lmd*, resulted in a robust expression of Lmd downstream transcriptional targets such as Vrp1 (Fig. 9A-B). At stage 13/14, VM expression of Vrp1 declines and is less prominent when compared with expression in the SM at this time. However, in embryos expressing *UAS-Lmd$^{141\text{-}866}$*, Vrp1 expression is robust in both the VM and SM (Fig. 9B,D; supplementary material Fig. S4, compare with Fig. 9A,C). Examination of the visceral muscle with Fas3 reveals that these embryos do not form a proper gut musculature (Fig. 9D′). The induction of Vrp1 expression by Lmd$^{141\text{-}866}$ can be clearly observed in all mesodermal tissue with *twistGal4*, and no detectable Fas3-positive visceral muscle is observed (Fig. 9E; supplementary material Fig. S4C).

Given these *in vivo* results, we examined the ability of Alk activity to regulate the subcellular localisation of the Lmd$^{141\text{-}866}$ mutant protein in cell culture. In contrast to the Lmd^{WT} protein or the Lmd^{PXXP} deletion mutant (in which residues 163-179 'PQTPYTPYTPYTPYTPC' are deleted), which are relocalised from the nucleus to the cytoplasm in response to Alk signalling, we observed that Alk was unable to efficiently relocate the Lmd$^{141\text{-}866\text{-}GFP}$ protein (Fig. 10A-E). In keeping with these findings, the Lmd$^{141\text{-}866}$ mutant protein was clearly present in visceral FCs (supplementary material Fig. S5A) as well as in stage 13 VM cells (Fig. 9A,B), at a time when endogenous, or ectopically expressed, wild-type Lmd protein was undetectable (Fig. 9; supplementary material Fig. S5). Thus, the Lmd$^{141\text{-}866}$ mutant that acts as a constitutively active protein in the developing *Drosophila* visceral muscle is also unresponsive to Alk signals that induce relocation from the nucleus to the cytoplasm in cell culture experiments. These data suggest that exclusion of Lmd protein from the nucleus plays an important role in the regulation of Lmd protein activity *in vivo*.

We next performed mass spectrometry analysis of Lmd to identify phosphorylation events in response to ALK activation. Analysis of Lmd-GFP from HEK293 cells led to the identification of multiple phosphorylated amino acids (serines 8, 18, 19, 21, 33 and 40, and tyrosine 35) in the N terminus of Lmd-GFP. In this analysis, we could confirm phosphorylation of the previously described putative PKA phosphorylation sites within the C-terminal region at residues 596 to 599 (RRHpS) and 617 to 620 (RRHpS) (Duan and Nguyen, 2006). Interestingly, the PxxP motif was not phosphorylated, confirming our findings that these residues are unlikely to be important for ALK-mediated regulation. Although the N-terminal region of Lmd is clearly important for ALK

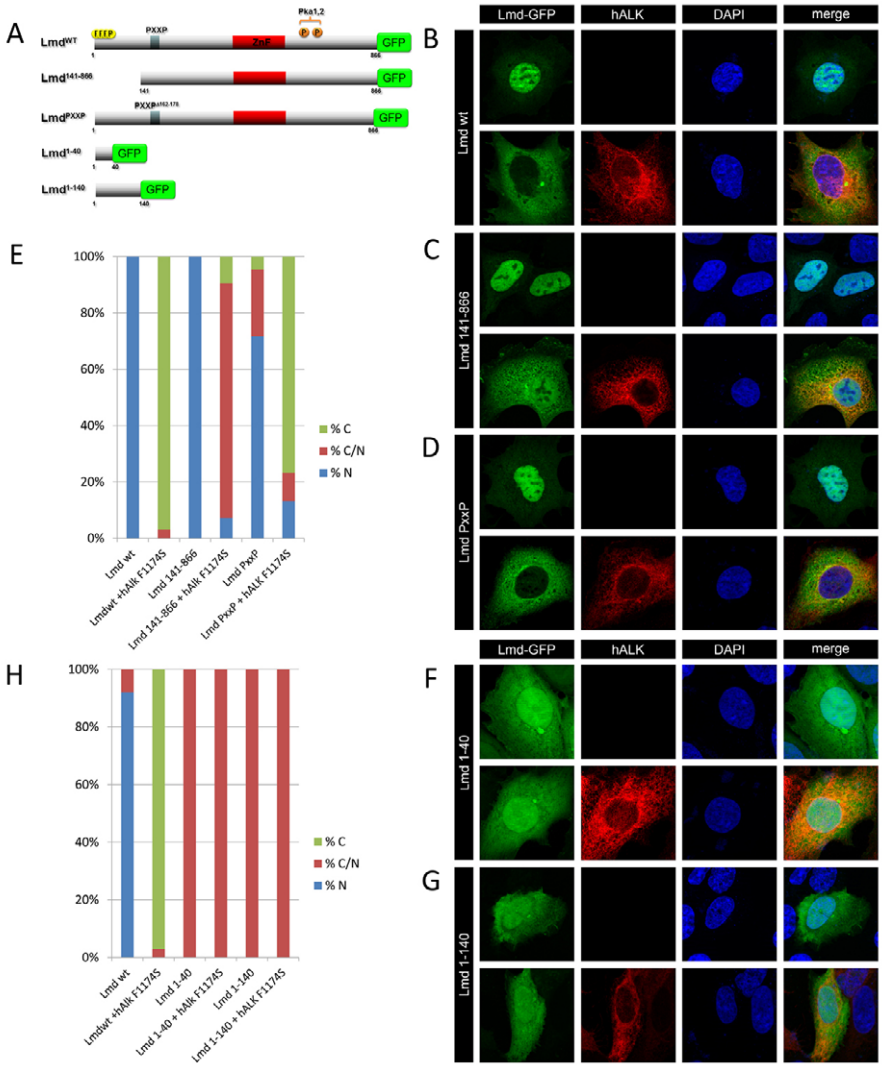

Fig. 10. The N-terminal region of Lmd is required, but not sufficient, for relocalisation of Lmd in response to Alk signalling. (**A**) Lmd-GFP fusions and phosphorylation sites identified by mass spectrometry analysis. (**B**) Lmd-GFP (green) is localised exclusively in nucleus (DAPI, blue) in absence of ALK activity (upper panel), whereas the presence of activated human ALK^{F1174S} leads to translocation of Lmd-GFP to the cytoplasm (lower panel). (**C**) The N-terminal Lmd-141-866-GFP truncation mutant is nuclear both in the absence (upper panel) and presence of activated human ALK^{F1174S} (lower panel). (**D**) Lmd-PXXP-GFP displays cytoplasmic localisation when activated human ALK^{F1174S} is present (lower panel). (**E**) Quantification of B-D (C, cytoplasmic; N, nuclear; C/N, cytoplasmic and nuclear). (**F**,**G**) Lmd-1-40-GFP (F) and Lmd-1-140-GFP (G) (green) are localised both in the nucleus and the cytoplasm (DAPI, blue) regardless of the presence of activated human ALK^{F1174S} (lower panel). (**H**) Quantification of F and G.

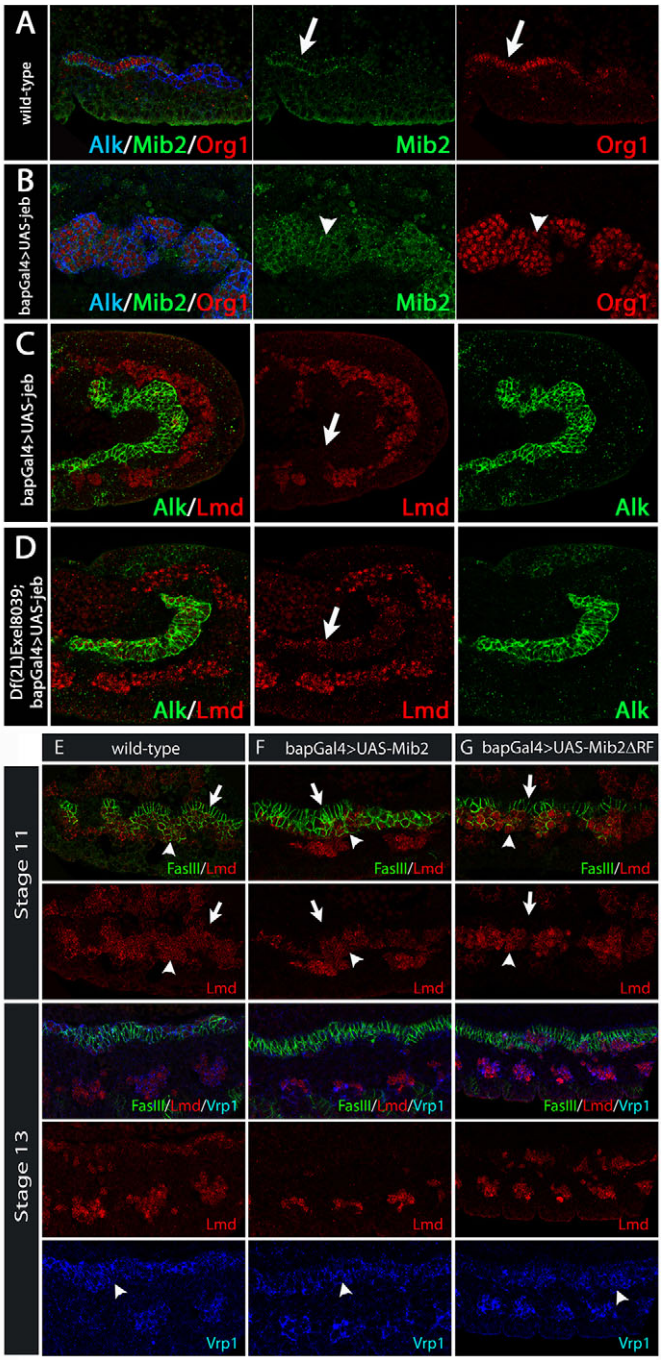

Fig. 11. Alk-mediated downregulation of Lmd requires Mib2 activity. (**A**) Mib2 (green) and Org-1 (red) are expressed in visceral FCs (arrows). Alk (blue) is expressed in FCs and FCMs. (**B**) Mib2 expression (green) is observed in all VM cells (arrowheads) upon ectopic expression of Jeb in *bapGal4>UAS-jeb* embryos. Similarly, Org-1 expression (red, arrowhead) is expanded to all Alk-positive (blue) VM cells. (**C**) Lmd protein (red) is undetectable in VM (arrow; Alk in green) of *bapGal4>UAS-jeb* embryos. (**D**) Downregulation of Lmd protein (red) is incomplete in VM (arrow, Alk in green) of *Df(2L)Exel8039*^{mib2}*;bapGal4>UAS-jeb* embryos. (**E**) In wild type, Lmd (red) is observed in visceral FCMs (arrowheads; stage 11; upper panels) and is downregulated after fusion (stage 13; lower panels). (**F**) Similar to control, FCMs (arrowheads) but not FCs (arrows) of *bapGal4>UAS-mib2* VM contain Lmd (red). (**G**) *bapGal4>UAS-mib2*$^{\Delta RF}$ visceral FCMs (arrowheads) express Lmd (red). To further assess Lmd in the VM, Vrp1 expression (blue, lower panels) was monitored at later stages in control (E), *bapGal4>UAS-mib2* (F) and *bapGal4>UAS-mib2*$^{\Delta RF}$ (G).

regulation of subcellular localisation, we wished to examine whether it was sufficient. To do this, we generated N-terminal Lmd-GFP fusions, corresponding to 1-40 and 1-140 residues of Lmd fused to GFP (Lmd$^{1\text{-}40\text{-}GFP}$ and Lmd$^{1\text{-}140\text{-}GFP}$, respectively) (Fig. 10A). Both display nuclear and cytoplasmic localisation, and were unaffected by ALK activity (Fig. 10F,G). Thus, we conclude that amino acids 1-140 of Lmd are not sufficient for nuclear to cytoplasmic relocation in response to ALK activity.

Alk coordinated Lmd downregulation by Mib2 in the VM

One protein described as involved in the regulation of Lmd in the somatic muscle is the Mind bomb2 (Mib2) ubiquitin ligase (Carrasco-Rando and Ruiz-Gómez, 2008). Interestingly, Mib2 is also expressed in visceral muscle, more specifically in FCs where it is transcriptionally controlled by Alk signalling (Fig. 11A,B). To test whether Mib2 plays a role in the downregulation of Lmd by Alk signalling in the VM, we examined the effect of Jeb overexpression in *mib2*-deficient *Df(2R)Exel8039* or *mib2*^{1}*/Df(2R)Exel8039* embryos. Overexpression of Jeb (*bapGAL4>UAS-jeb*) leads to a loss of Lmd in the VM in the presence of wild-type levels of Mib2 (Fig. 11C). However, in a *mib2* mutant background, Lmd protein is still observed (Fig. 11D), supporting a role for Mib2 in VM degradation of Lmd. This is in keeping with previous findings, showing that Lmd protein persists in the somatic musculature of *mib2* mutants (Carrasco-Rando and Ruiz-Gómez, 2008). As Mib2 is specifically expressed in the FCs, we tested whether regulation of Mib2 expression in visceral FCs is sufficient for the loss of Lmd protein in the VM. To do this, we employed *UAS-Mib2* transgenes (Nguyen et al., 2007) to ectopically express Mib2 in the VM and subsequently analyse Lmd protein levels and activity. Expression of either *UAS-Mib2* or the *UAS-Mib2*$^{\Delta RF}$ deletion mutant (in which the C-terminal RING finger domains are deleted) did not lead to an ectopic loss of Lmd protein in the VM (Fig. 11E-G). Lmd is present in FCMs of control, *bapGAL4>UAS-Mib2* and *bapGAL4>UAS-Mib2*$^{\Delta RF}$ embryos, and absent in FCs and fused VM. To confirm the presence of active Lmd protein, we monitored Vrp1 expression in the VM at later stages (Fig. 11E-G, lower panel). Although Lmd protein appeared to be lost more rapidly upon Mib2 expression, when compared with Mib2$^{\Delta RF}$, Vrp1 expression was clearly seen in all cases, suggesting that simple expression of Mib2 is not sufficient for Lmd protein loss. These data support a model in which Alk activity not only regulates transcription of Mib2, but is also required for modification of Lmd, which then allows recognition by Mib2 (or a Mib2-containing complex), subsequently leading to a loss of Lmd protein.

Taken together, our results suggest a model in which Alk signalling leads to the relocation of Lmd protein from the nucleus to the cytoplasm in response to Jeb activation of Alk and upon fusion of FCs with FCMs. Once extruded from the nucleus, potentially carrying post-translational modifications, Lmd-mediated transcriptional regulation, illustrated here by the Lmd target gene *Vrp1*, is abrogated and Lmd is accessible to the action of the Mib2 E3 ligase and to the subsequent degradation that promotes reprogramming of FCMs (Fig. 12).

DISCUSSION

Although the role of Jeb/Alk in FC specification in the *Drosophila* VM is well studied, little is known about target molecules that mediate Alk signalling-dependent transcription. Such molecules should possess activating or repressing DNA-binding transcription factor activity or function as co-factors for transcription factors.

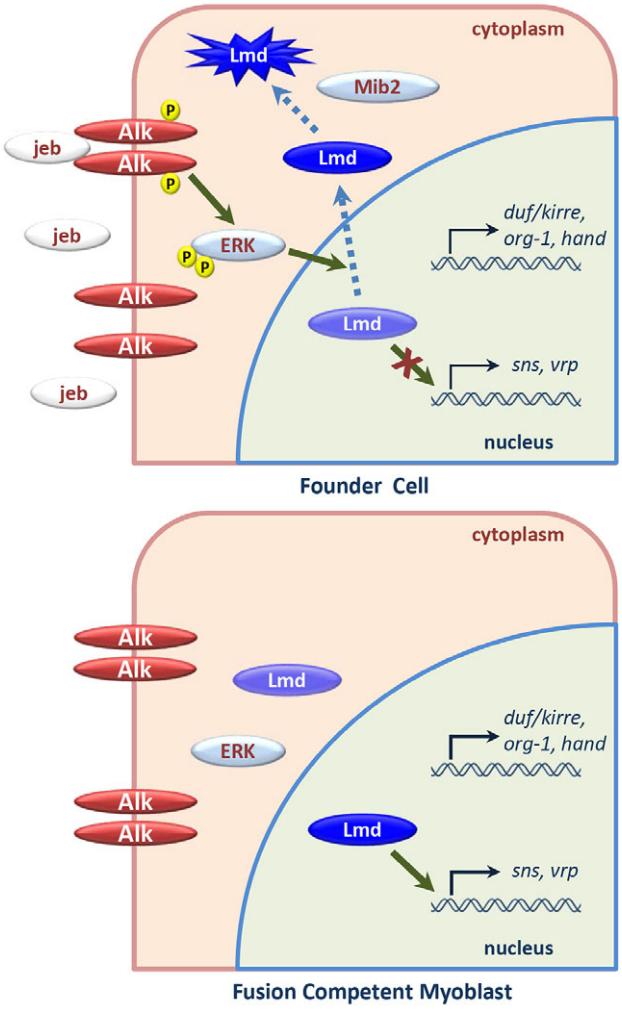

Fig. 12. Model for the Alk-mediated downregulation of Lmd in the developing VM. The Jelly belly (Jeb) ligand binds to Alk in the VM. Activated Alk initiates signal transduction in visceral FCs resulting in subsequent nuclear to cytoplasmic translocation of Lmd protein, where it becomes accessible to degradation by the Mib2 ubiquitin ligase. In FCMs, lack of Alk signaling results in Lmd-mediated expression of FCM-specific genes.

Among the known transcriptional targets of the Jeb/Alk pathway expressed in the VM are the transcription factors Org-1 (Lee et al., 2003) and Hand (Varshney and Palmer, 2006). Bagpipe (Bap), Biniou (Bin) and Lmd are additional transcriptional factors expressed in the VM (Azpiazu and Frasch, 1993; Duan and Nguyen, 2006; Ruiz-Gómez et al., 2002; Zaffran et al., 2001). However, neither expression of Bap or Bin protein nor that of several direct targets of these transcription factors (such as Fas3) is affected in the VM of *alk* mutants. Activation of Alk, in all cells of the VM leads to the downregulation of FCM-specific genes such as *sns*, and an upregulation of FC-specific genes such as *duf/kirre*, *org-1* and *hand*. Lmd is known to play a critical role in FCM specification in the somatic mesoderm of *Drosophila* embryos (Duan et al., 2001; Furlong et al., 2001; Ruiz-Gómez et al., 2002), and our data show an important regulatory role for Lmd also in the VM.

It is interesting that the FCMs in the VM, converted to 'founder-like' cells in either *lmd* mutants or *bapGal4>UAS-jeb* embryos, are able to contribute to a semi-functional gut musculature, albeit the number of nuclei and the proportions of the different visceral muscle types are clearly altered. This resembles the loss of Lmd in the somatic mesoderm, whereupon FCMs fail to mature and convert to pericardial and APM-like cells (Sellin et al., 2009). One possible explanation may involve the key myogenic factor Mef2, which is a target for Lmd regulation. In somatic FCMs, Lmd directly regulates Mef2, whereas in VM, expression of Mef2 appears to be independent of Lmd activity (Duan et al., 2001; Furlong et al., 2001). This intriguing difference could explain why converted FCMs in the VM of *lmd* mutants are able to differentiate into myotubes.

FCM progenitors in *lmd* mutant embryos fail to express FCM-specific molecules required for muscle fusion, such as Sns and Vrp1, whereas expression of the FC-specific transcription factors Hand and Org-1 is expanded to all cells of the VM. Interestingly, activation of Alk signalling in all VM cells mimics the $lmd^{-/-}$ mutant phenotype in terms of FC gene expression and increased cell proliferation. Taking these data into consideration, the modulation of the Lmd transcription factor is an attractive candidate target for Jeb/Alk signalling in the developing embryonic *Drosophila* VM.

In our experiments, we show that Lmd is indeed a direct regulatory target of *Drosophila* Alk signalling *in vivo*, in the VM. These findings are supported by cell culture experiments in which Lmd-GFP undergoes nuclear cytoplasmic translocation when *Drosophila* Alk is activated by Jeb. In developing VM, activation of Alk signalling leads to phosphorylation of MAPK (ERK), suggesting that downregulation of Lmd protein *in vivo* may occur via translocation of phosphorylated Lmd to the cytoplasm from the nucleus, as a result of activation of the MEK signalling cascade. Whether Lmd phosphorylation is a direct or indirect result of MAPK (ERK) activity is currently unclear and requires further investigation. The modulation of Lmd by *Drosophila* Alk could be extended to human *ALK* where, $hALK^{F1174S}$, a constitutively active mutation observed in the childhood cancer neuroblastoma (Martinsson et al., 2011) is also able to regulate subcellular localisation of Lmd.

Previously, the Lmd protein sequence was dissected to search for functional elements that are able to modulate Lmd transcriptional activity (Duan and Nguyen, 2006). In the present study, we have examined a number of mutant forms of Lmd and identified one mutant protein – in which the first 140 amino acids are deleted – that does not respond to active Alk signalling. Interestingly, deletion of a number of potential MAPK (ERK) phosphorylation sites in the N-terminal region of Lmd, represented by the Lmd^{PXXP} mutant (in which residues 163-179 'PQTPYTPYTPYTPYTPC' are deleted), did not affect the ability of Alk to modulate Lmd subcellular organisation, and indeed these were not phosphorylated in our mass spectrometry analysis. However, amino acids 1-140 of the Lmd protein contain multiple phosphorylation sites identified by mass spectrometry, some of which appear to be completely conserved throughout the *Drosophila* species sequenced to date.

In light of these results, we analysed the importance of the N-terminal region of Lmd, but were unable to observe ALK-regulated translocation of these short regions of Lmd in cell experiments, suggesting that the context of the full-length protein is required for translocation. This is perhaps not surprising as amino acids 1-140 of Lmd lack known functional domains, and key elements required for regulation by ALK activity might be missing. Although insensitive to regulation by ALK, the $Lmd^{141\text{-}866}$ mutant lacking the N-terminal 140 amino acids was still able to regulate downstream genes, robustly driving Vrp1 expression. Thus, this short N-terminal region of the Lmd protein is important for efficient relocalisation of Lmd protein to the cytoplasm from the nucleus. Further experiments will be required to determine which other parts of Lmd are also involved.

Notably, although *lmd* mRNA can been observed in visceral FCs of *Drosophila* embryos, we did not detect Lmd protein in these cells. Post-translational modifications are a well-described phenomenon in the regulation of many transcription factors, and some interesting comparisons can be drawn with work on *Drosophila* Yan. Yan is an ETS transcription factor family member that acts downstream of RTK signalling as a transcriptional repressor and negative regulator of cell differentiation during eye development. Interestingly, Yan is regulated via post-translational modifications in a manner similar to our observations with Lmd, undergoing phosphorylation in response to RTK/Ras/MAPK (ERK) signalling cascade activity and is subsequently exported from the nucleus to the cytoplasm, where it is finally degraded (Hsu and Schulz, 2000; Lai and Rubin, 1992; Mavrothalassitis and Ghysdael, 2000; Rebay and Rubin, 1995; Rogge et al., 1995; Roukens et al., 2008; Tootle et al., 2003). Furthermore, F-box-mediated ubiquitylation of Yan has been reported to promote its downregulation (Roukens et al., 2008), with similarities to the proposed regulation of Lmd by the Mind bomb 2 ubiquitin ligase. Interestingly, in addition to identifying multiple phosphorylation sites in Lmd, we also identified ubiquitin modification of lysine 6 in the N-terminal region of Lmd in our mass spectrometry analysis. Whether this observed ubiquitylation has *in vivo* relevance will require more detailed analysis.

The identification of the E3 ligase Mind bomb 2 (Mib2) and its role in myogenesis, provides an elegant link to the fate of the Lmd protein. It has been suggested that Mib2 plays a role in the degradation of Lmd in the somatic mesoderm (Carrasco-Rando and Ruiz-Gómez, 2008; Nguyen et al., 2007), supporting our finding that it has a similar role in the VM, which is facilitated by Alk signalling. The data presented herein indicate that Alk signalling results in Lmd protein modification and exclusion from the nucleus, where it is then accessible for Mib2-targeted degradation. Moreover, from the data presented here, it is convincing that Mib2 expression is itself regulated by Jeb/Alk signalling activity in the VM, thus imparting an additional layer of regulation.

In mammalians, members of GLI family of transcription factors, such as GLI1, also undergo post-transcriptional modification via MEK signalling (Schnidar et al., 2009). However, in this case, GLI1 is activated by RAS/MEK pathway by relocating to the nucleus from the cytoplasm. In fact, the mammalian GLI family comprises the GLI, GLIS and ZIC proteins, with Lmd displaying highest homology with the lesser studied GLIS proteins subfamily (Kang et al., 2010). In the childhood cancer neuroblastoma, in which human ALK is implicated, GLI signalling has not been well studied. It will be interesting to examine the regulation of GLI signalling, or that of the related GLIS and ZIC proteins, in the context of human ALK activity in neuroblastoma.

Taken together, our data show that the Lmd transcription factor is a target of Jeb/Alk signalling in the VM of *Drosophila* embryos. Whether this mechanism is employed during other Alk regulated processes in *Drosophila*, or in neuroblastoma progression in humans will require further investigation.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
D.P. and R.H.P. designed the experiments and wrote the paper. D.P. conducted the majority of the experiments. F.H., C.S., M.D., Y.Y., G.W., C.S., M.F. and R.H.P. also contributed to the experimental analysis. B.H. and H.N. were involved with the experimental design and analysis. All authors were involved in the final stages of writing the article.

Supplementary material
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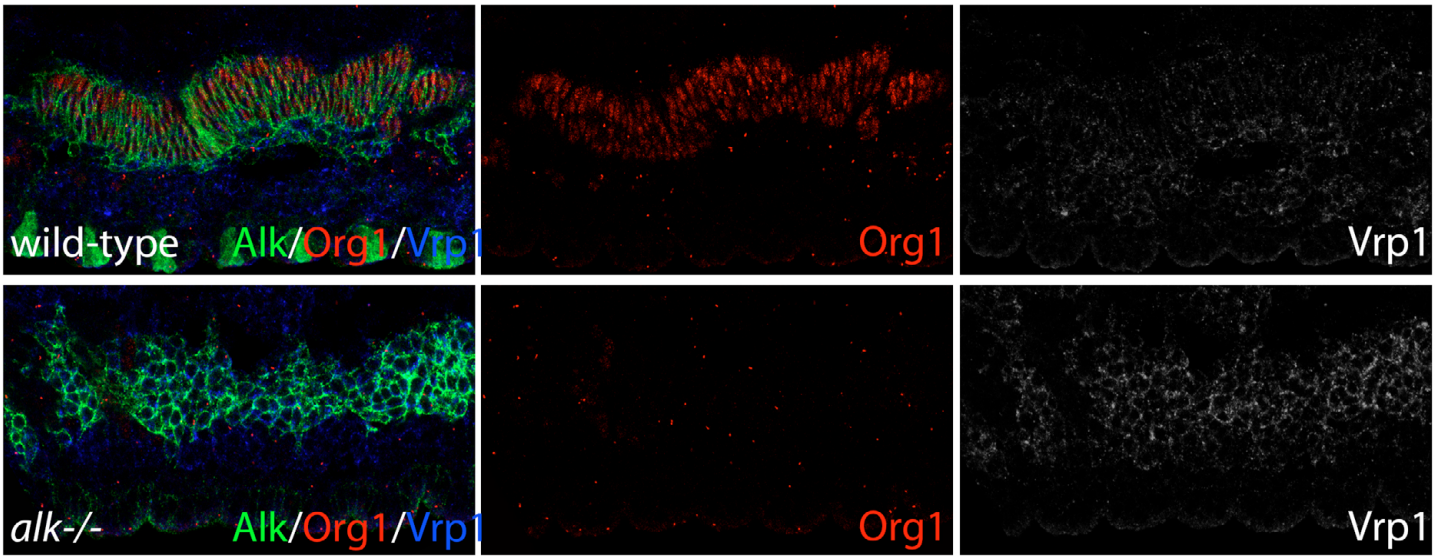

Fig. S1. *alk* mutant VM cells are Org-1 negative and Vrp1 positive. (Upper panel) In wild-type embryos (stage 13), Org-1 protein (red) is observed in the fusing cells of the VM. All VM cells express Alk (green). (Lower panel) Org-1 protein (red) expression is absent in VM cells of *alk*$^{-/-}$ embryos. Vrp1-positive cells denoted in white or blue (merged panel).

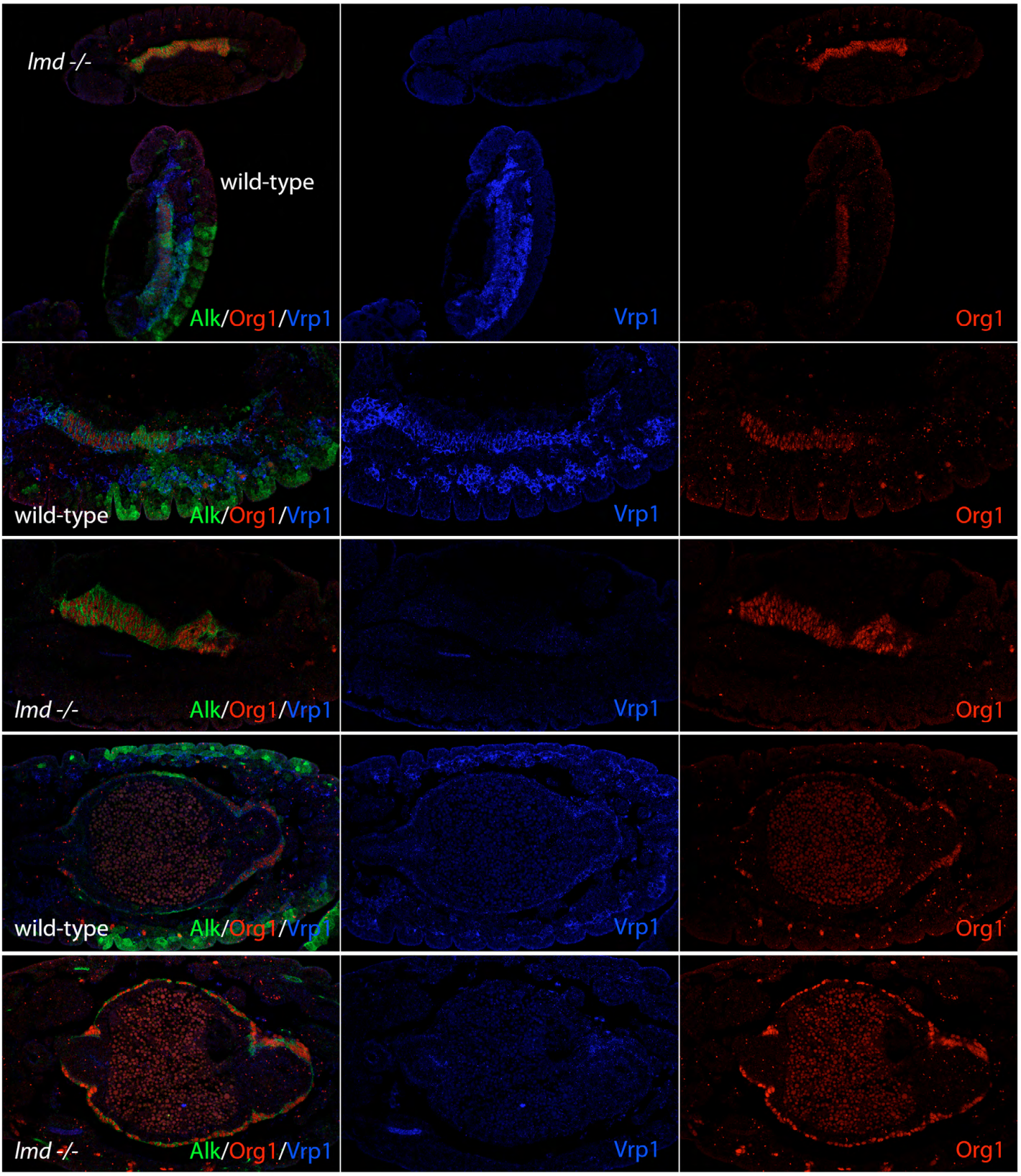

Fig. S2. VM in *lmd* mutants fail to express Vrp1 protein at later stages of embryonic development. In wild-type embryos, Org-1 protein (red) is observed in the fusing cells of the VM, together with Vrp1 protein (blue). All VM cells express Alk (green). VM cells in *lmd*$^{-/-}$ embryos (marked with Alk in green) robustly express Org-1 (red) but lack Vrp1 protein (blue).

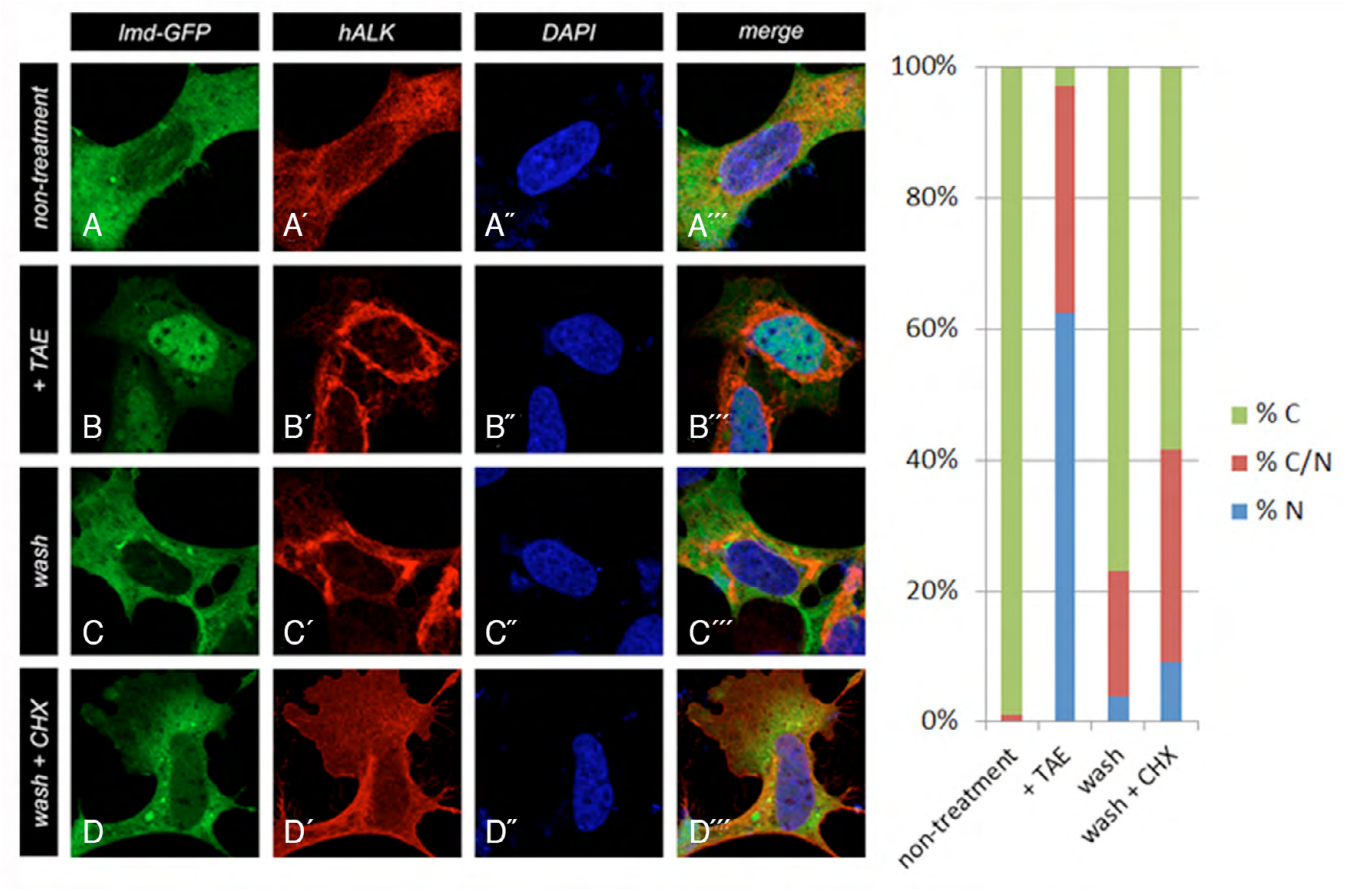

Fig. S3. ALK activity-dependent Lmd translocation does not require *de novo* protein synthesis. HEK293 cells transfected with Lmd-GFP and gain-of-function human ALK^{F1174S} were pretreated with 0.2 µM NVP-TAE684, a specific ALK inhibitor, for 18 hours. Following a 2-hour pretreatment with 50 µg/ml cycloheximide (CHX) to block *de novo* protein synthesis, NVP-TAE684 was washed away and cells were further incubated for 30 minutes in the presence of CHX. ALK was visualized by immunostaining (red) and nuclei marked by DAPI (blue). (**A**,**B**) Translocation of Lmd protein (green) to cytosol in non-treated cells (A) is blocked in the presence of NVP-TAE684 (B). (**C**,**D**) After removal of ALK inhibitor, Lmd protein re-distributes to cytosol (C), even in the presence of CHX (D). Subcellular localisation of Lmd protein is quantified on the right. C, cytosol; N, nucleolus; C/N, both.

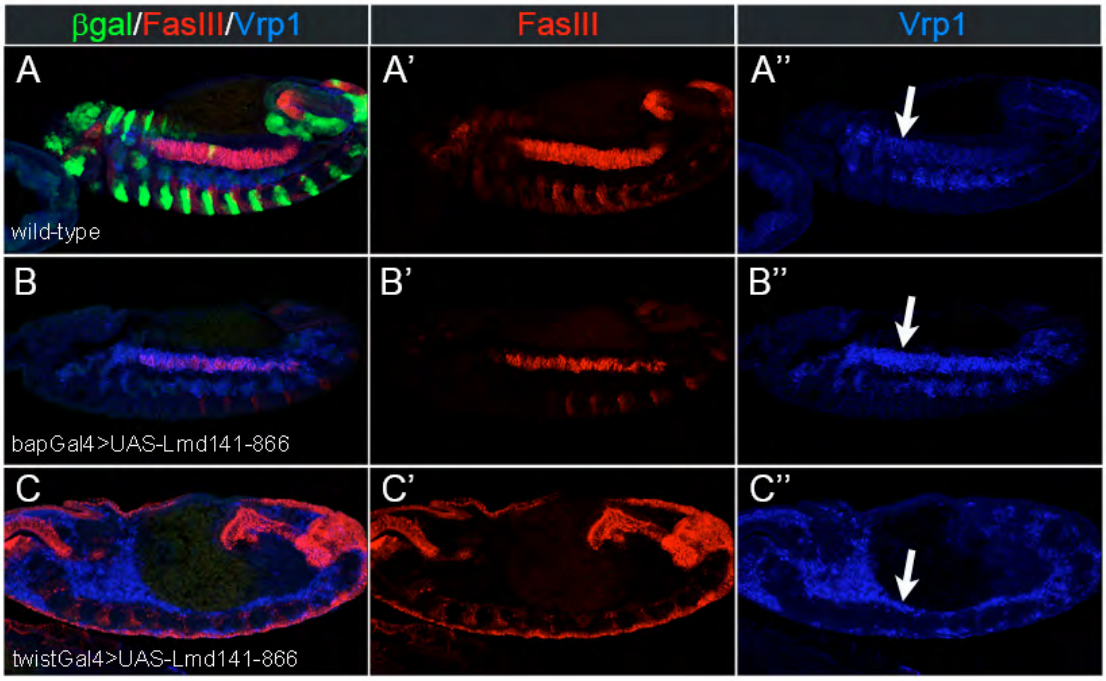

Fig. S4. Expression of the Lmd$^{141-866}$ mutant protein in the mesoderm drives robust expression of Vrp1. (**A-A″**) In wild-type embryos (stage 13), Vrp1 protein (blue) is observed in the FCM of the VM and is downregulated after fusion (arrow). (**B-B″**) All VM cells in *bapGal4>UAS-lmd$^{141-866}$*-expressing embryos (marked with Fas3 in red) express high levels of Vrp1 (blue, arrow), which is not downregulated. (**C-C″**) In *twistGal4>UAS-lmd$^{141-866}$*, both VM and SM express high levels of the Lmd target Vrp1 (blue, arrow). No Fas3-positive VM can be observed (red).

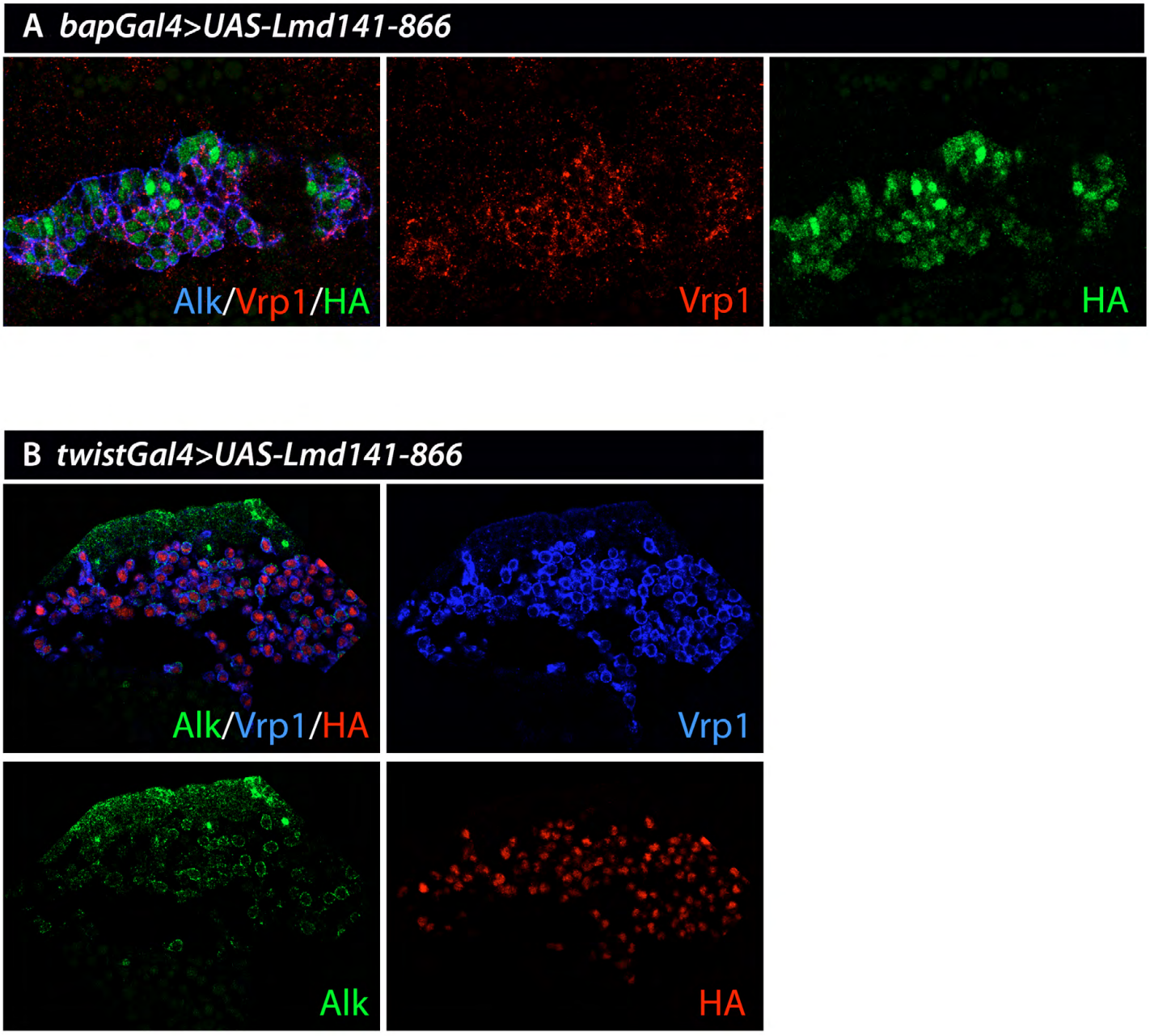

Fig. S5. The Lmd$^{141-866}$ mutant protein persists in Alk positive VM cells. (**A**) VM FCs in *bapGal4>UAS-lmd$^{141-866}$*-expressing embryos (marked with Alk in blue) express Vrp1 (red). Both FCs and FCMs display nuclear Lmd$^{141-866}$ mutant protein (HA in green). (**B**) In *twistGal4>UAS-lmd$^{141-866}$*, both visceral and somatic mesoderm persistently express nuclear Lmd$^{141-866}$ mutant protein (HA in green). This is associated with high levels of expression of the Lmd target Vrp1 (blue) in these cells. In this case, a subset of the Vrp1, Lmd-positive cells are Alk positive (green).