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STEM CELLS AND REGENERATION

RESEARCH REPORT

Nutrition and PI3K/Akt signaling are required for p38-dependent regeneration

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ABSTRACT

Regeneration after damage requires early signals to trigger the tissue repair machinery. Reactive oxygen species (ROS) act as early signals that are sensed by the MAP3 kinase Ask1, which in turn activates by phosphorylation the MAP kinases p38 and JNK. The sustained or high activation of these kinases can result in apoptosis, whereas short or low activation can promote regeneration. Using the Ask1-dependent regeneration program, we demonstrate in *Drosophila* wing that PI3K/Akt signaling is necessary for Ask1 to activate p38, but not JNK. In addition, nutrient restriction or mutations that target Ser83 of the *Drosophila* Ask1 protein, a PI3K/Akt-sensitive residue, block regeneration. However, these effects can be reversed by the ectopic activation of p38, but not of JNK. Our results demonstrate that Ask1 controls the activation of p38 through Ser83, and that the phosphorylation of p38 during regeneration is nutrient sensitive. This mechanism is important for discriminating between p38 and JNK in the cells involved in tissue repair and regenerative growth.

KEY WORDS: *Drosophila*, Growth, Insulin, Regeneration, Signaling

INTRODUCTION

One of the most exciting questions in regenerative biology is how damaged cells signal to their surviving neighbors to stimulate tissue repair (Hariharan and Serras, 2017). The stress-activated MAP kinases Jun N-terminal kinase (JNK) and p38 respond to the oxidative stress generated by damaged cells (Diwanji and Bergmann, 2018; McCubrey et al., 2006; Serras, 2016). These multitasking kinases regulate a variety of cell functions, including healing, growth and apoptosis (Martínez-Limón et al., 2020). Several MAP3 kinases are known to operate upstream of JNK and p38 (Sakauchi et al., 2017; Son et al., 2013; Takeda et al., 2008). Apoptosis signal-regulating kinase 1 (Ask1), a MAP3 kinase that, upon oxidative stress, oligomerizes to become activated (Sakauchi et al., 2017), has emerged as a potential signal in the damage response in *Drosophila* epithelia (Patel et al., 2019; Santabárbara-Ruiz et al., 2019; Toshniwal et al., 2019). In its inactive form, Ask1 is bound to its inhibitor thioredoxin (Saitoh et al., 1998). Upon oxidative stress, thioredoxin dissociates from the thioredoxin-binding domain of Ask1, enabling Ask1 to oligomerize and its threonine-rich kinase domain to autophosphorylate, leading to its activation and function as a MAP3 kinase (Fig. 1A) (Liu et al., 2000; Nishida et al., 2017; Saitoh et al., 1998).

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Received 17 September 2020; Accepted 29 March 2021

Genetically induced cell death in *Drosophila* imaginal discs results in oxidative stress that activates p38 and JNK, triggering regeneration (Brock et al., 2017; Fan et al., 2014; Fogarty et al., 2016; Fox et al., 2020; Herrera and Morata, 2014; Herrera et al., 2013; Khan et al., 2017; Santabárbara-Ruiz et al., 2015, 2019; Serras, 2016). In this context, Ask1 acts upstream of p38 and JNK, and is sensitive to reactive oxygen species (ROS) (Patel et al., 2019; Santabárbara-Ruiz et al., 2019). Phosphorylation of p38 is an early response to damage that occurs alongside JNK (Santabárbara-Ruiz et al., 2019). However, JNK can also trigger apoptosis (Pinal et al., 2019). How increased p38 phosphorylation can coexist with tolerable, presumably low, levels of JNK remains unclear.

There are many observations that support an antagonism between p38 and JNK, mainly reinforced by the finding that p38 can negatively regulate JNK activity in mammalian cells (Wagner and Nebreda, 2009). For example, the inhibition of p38 in mammalian myoblasts, epithelial cells and macrophages leads to the activation of JNK (Cheung et al., 2003; Perdiguero et al., 2007). Moreover, Jun-deficient hepatocytes show increased p38α phosphorylation (Stepniak et al., 2006). Therefore, we speculated that the mechanism that activates p38 during *Drosophila* regeneration operates concomitantly with a reduction of JNK. As MAP3 kinases are key regulators of MAPK activity, we decided to investigate how Ask1 discriminates between p38 and JNK, and particularly how Ask1 fuels p38-dependent regeneration.

We have previously shown that the Ser83 residue of the *Drosophila* Ask1 protein is required for tissue repair, and that phosphorylation of Ask1 at Ser83 depends on PI3K/Akt signaling (Santabárbara-Ruiz et al., 2019). Insulin signaling through the PI3K/Akt pathway indicates the nutritional status of an animal and regulates tissue growth (Hietakangas and Cohen, 2009). Here, we present evidence that the Ser83 residue of Ask1 is key for activating p38, but not JNK; therefore, discriminating between p38 and JNK. Moreover, we show that nutrients and PI3K/Akt signaling are necessary for p38 activation in cells involved in regeneration

RESULTS AND DISCUSSION

Activation of p38 but not JNK requires the Ser83 residue of Ask1

The YH_GVRESF sequence located in the N-terminal region of Ask1 is highly conserved from sponges to humans (Fig. 1A and Fig. S1A). This sequence is present in the Ask1-PC isoform of *Drosophila* and contains the Ser83 residue (Ser174 in humans and Ser181 in mouse). Phosphorylation of Ser83 by Akt is required to control Ask1 kinase activity, occurring in the same cells that activate p38 during regeneration (Santabárbara-Ruiz et al., 2019). This prompted us to hypothesize that Ser83 of the Ask1 protein could be essential for p38 activation.

We have previously demonstrated that the ectopic activation of wild-type Ask1 (*UAS-Ask1^{WT}*) resulted in a moderate increase in Ask1 activity in the absence of extra thioredoxin inactivation

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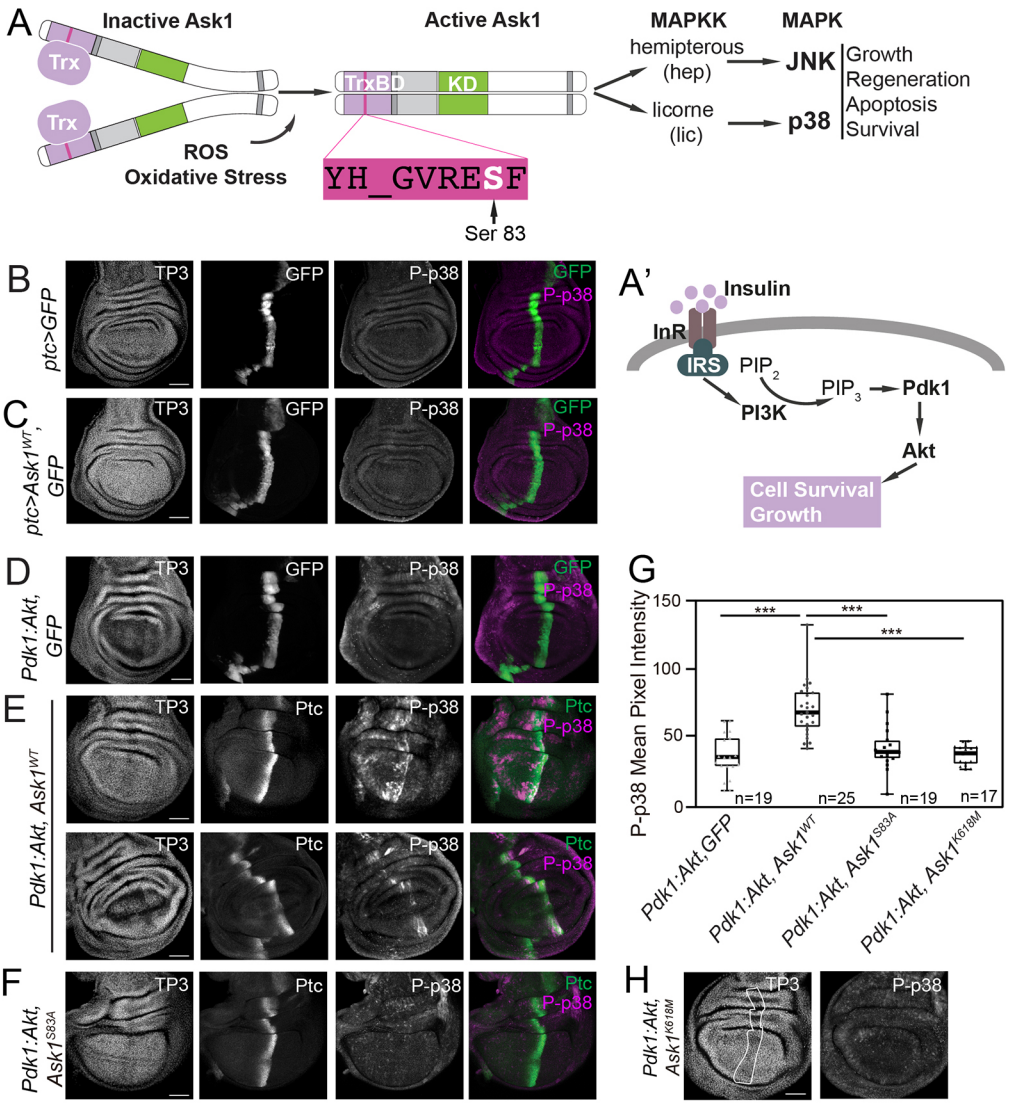

Fig. 1. The co-expression of *Ask1* and *Pdk1:Akt* results in the activation of p38. (A) Ask/p38/JNK signaling. (A′) Insulin/PI3K/Akt signaling. (B-D) P-p38 in (B) GFP-expressing control, (C) wild-type $Ask1^{WT}$, and (D) *Pdk1:Akt* and *GFP*. (E) Two examples of P-p38 after co-expressing *Pdk1:Akt* and $Ask1^{WT}$. (F) P-p38 in *Pdk1:Akt* and $Ask1^{S83A}$. (G) Mean pixel intensity of P-p38 for the genotypes indicated. Box plots show maximum-minimum range (whiskers), upper and lower quartiles (open rectangles), and median value (horizontal black line). ***P<0.001. (H) P-p38 after *Pdk1:Akt* and $Ask1^{K618M}$ co-expression. White line in H indicates area of *ptc*>expression. TP3 is used to stain nuclei. Scale bars: 50 µm.

(Santabárbara-Ruiz et al., 2019). Ectopic expression of $Ask1^{WT}$ in wing imaginal discs using the *patched-Gal4* driver (hereafter *ptc*>) did not increase phosphorylated p38 (P-p38) levels beyond basal values (Fig. 1B,C). As the sole expression of *Ask1* is not enough to phosphorylate p38, we decided to determine whether p38 phosphorylation requires Akt and the Ser83 residue of Ask1. We used *ptc-Gal4* to drive the expression of three transgenic combinations and tested them for p38 activation, as follows.

(1) In the control experiments, we ectopically expressed *Akt* and *GFP*. To activate Akt, we used the *UAS-Pdk1:UAS-Akt1* recombinant construct (hereafter *Pdk1:Akt*). This transgene allows co-expression of Akt and *phosphoinositide-dependent kinase-1* (*Pdk1*), the gene encoding the kinase downstream of the insulin pathway that phosphorylates and activates Akt1 (Fig. 1A′). This construct ensures the ectopic expression of both genes and the activation of Akt without killing the cell. In this transgenic combination, we co-expressed *Pdk1:Akt* with *UAS-GFP* as a neutral transgene.

(2) One of the experimental conditions consisted of the ectopic expression of *Pdk1:Akt* and the wild-type form of Ask1 ($Ask1^{WT}$) to test the activation of Ask1 by Akt. (3) The other experimental condition consisted of the co-expression of *Pdk1:Akt* and the mutant $Ask1^{S83A}$, which cannot undergo Akt-dependent phosphorylation at Ser83. We first tested whether Akt was active in these combinations using an antibody against phospho-Akt (P-Akt). This revealed that P-Akt concentrated in the *ptc* stripe with similar intensities in all three transgenic conditions (Fig. S1B-E).

We found that P-p38 did not significantly increase in the *ptc* stripe of cells when *Pdk1:Akt* was co-expressed with *GFP* (Fig. 1D). Instead, P-p38 significantly increased when *Pdk1:Akt* was co-expressed with $Ask1^{WT}$ (Fig. 1E,G). P-p38 was highly concentrated in the *ptc*>stripe, although some labeling could also be observed in the cells anterior to the stripe. It is conceivable that this anterior staining resulted from the persistence of the early *ptc-Gal4* expression in the anterior compartment rather than a non-autonomous effect from dying cells, as no or only a few dead cells (positive for caspase Dcp1) were found (Fig. S2A-C) (Bosch et al., 2016; Evans et al., 2009). By contrast, P-p38 did not increase when *Pdk1:Akt* was co-expressed with the mutant $Ask1^{S83A}$, either in the *ptc*>stripe or in the anterior compartment (Fig. 1F,G). Therefore, we concluded that the Ser83 of the YH_GVRESF sequence is essential for p38 activation in PI3K/Akt signaling.

The same genotypes were tested with an antibody that recognizes the activated and phosphorylated form of JNK (Fig. S2E,F), observing that the basal levels did not respond to the ectopic expression of *Pdk1:Akt,* the co-expression of *Pdk1:Akt* and Ask^{WT},

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or the co-expression of *Pdk1:Akt* and *Ask1*^{S83A} (Fig. 2A-E). This was confirmed by analyzing matrix metalloproteinase 1 (MMP1), a known target of JNK signaling, which was only found to be associated with the very few apoptotic cells generated by the ectopic expression of the transgenes (Fig. S2A-D). These results demonstrated that the Akt-dependent phosphorylation of Ask1 at Ser83 is canalized through p38 and not through JNK, suggesting that the Ask1-dependent activation of JNK requires factors other than Akt.

The catalytically inactive *Ask1*^{K618M} mutant acts as a dominant-negative form that prevents phosphorylation of JNK (Kuranaga et al., 2002). We found that the increase of P-p38 in the discs co-expressing *Pdk1:Akt* and *Ask1*^{WT} could not be reproduced after the co-expression of *Pdk1:Akt* and the *Ask1*^{K618M} mutant (Fig. 1G,H). In addition, the co-expression of *Pdk1:Akt* and *Ask1*^{K618M} did not elicit significant changes in P-JNK levels (Fig. 2E,F). The activity of Akt, as tested with the anti-P-Akt antibody, in the discs co-expressing *Pdk1:Akt* and *Ask1*^{K618M} was similar to that of the other transgenic combinations (Fig. S1B-E,J). In conclusion, the inactive form of the Ask1 protein results in neither p38 nor JNK phosphorylation.

Ask1 activity was determined with an anti-P-Thr Ask1 antibody: a phospho-threonine antibody that targets the core of the Ask1 catalytic domain. This antibody showed increased staining in the discs co-expressing *Pdk1:Akt* and *Ask1*^{S83A} compared with the *Pdk1:Akt*, *Ask1*^{WT} and *Pdk1:Akt, Ask1*^{K618M} discs (Fig. S1F-I,K). The co-expression of *Pdk1:Akt* and *Ask1*^{WT} resulted in a very weak staining, although this was not statistically significant, suggesting that the ectopic expression of Akt attenuated P-Thr Ask1 levels (Fig. S1G). These results are consistent with previous observations showing a moderate increase in P-Thr Ask1 levels in *UAS-Ask1*^{WT}-expressing cells and a strong increase in P-Thr Ask1 levels in the *Ask1*^{S83A}-expressing cells, which concur with the role of Ser83 in the attenuation of Ask1 activity (Santabárbara-Ruiz et al., 2019). We propose here that this attenuation results in low levels of Ask1 activity that are still sufficient to activate p38. This is important for cell survival, as strong stimulation of Ask1 can result in apoptosis (Kuranaga et al., 2002). Together, these observations indicate that the activation of p38, but not JNK, is the result of the cooperation between the Ask1 kinase domain and the Ser83 residue of the YH_GVRESF sequence, which is tightly dependent on PI3K/Akt signaling.

Ectopic activation of p38 signaling rescues the defective regeneration caused by nutrient restriction

As P-p38 depends on Akt, we decided to study the role of p38 in regeneration under conditions of nutrient restriction. To trigger regeneration, genetic ablation was induced using the modified version of the LexA (LHG) transactivator system that can be conditionally controlled with the temperature-sensitive *Gal80*^{TS} (Yagi et al., 2010). We used the wing-specific *sal*$^{E/Pv}$ enhancer to drive the expression of LHG in the cells of the central part of the wing disc where the pro-apoptotic construct *lexO-rpr* was activated (*sal*$^{E/Pv}$*-LHG lexO-rpr)*. In addition, we used the transactivator Gal4/UAS to drive the expression of a second transgene (*UAS-GFP*, *UAS-lic*^{WT} or *UAS-hep*^{WT}) in the same individuals (Fig. 3A). The expression of any of these three transgenes driven by *nub-Gal4* in unablated discs did not affect the normal pattern of the wings, although a small reduction in size was detected for *UAS-lic*^{WT} and *UAS-hep*^{WT} in comparison with the control *UAS-GFP* (Fig. S3).

We first analyzed the wing imaginal discs of animals grown in standard food conditions (100% yeast concentration) in which we induced apoptosis (*sal*$^{E/Pv}$*-LHG lexO-rpr*) and activated a neutral transgene (*nub-Gal4 UAS-GFP)*. In these animals, wing discs showed P-p38 surrounding the apoptotic domain (Fig. 3B). Adult animals emerged with normally regenerated wings in 97% of the females and 89% of the males, suggesting a high regeneration capability (Fig. 3E,F). In the controls kept at 17°C to block cell death, 100% of the wings were normal (Fig. 3G,H). The dimorphic phenotype could be due to the fact that females initiate metamorphosis at a larger body size than males and, in addition, females grow faster than males during the last phase of larval growth (Testa et al., 2013).

However, under conditions of nutrient restriction (10% yeast), P-p38 was absent in the wing discs (Fig. 3I) and the percentage of normally regenerated wings dropped to 67% in females and 50% in males (Fig. 3L,M). These animals had smaller wings (Fig. 3E,F,L, M) and showed a lack of veins and interveins as well as missing sectors of the wing (Fig. 3N,O). These results demonstrated that patterning and size of regenerated wings were affected by the

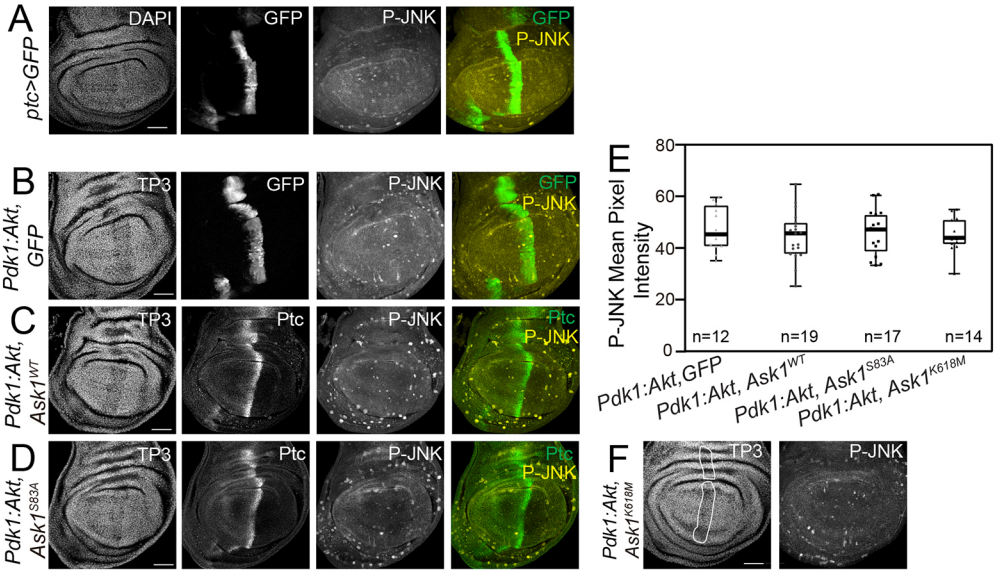

Fig. 2. The co-expression of *Ask1* and *Pdk1:Akt* does not result in the activation of JNK. (A) P-JNK in GFP-expressing control. (B-D) P-JNK afer co-expression of (B) *Pdk1:Akt* and *GFP*, (C) *Pdk1:Akt* and *Ask1*^{WT}, or (D) *Pdk1:Akt* and *Ask1*^{S83A}. (E) Mean pixel intensity of P-JNK for the genotypes indicated. Box plots show maximum-minimum range (whiskers), upper and lower quartiles (open rectangles), and median value (horizontal black line). (F) P-JNK after co-expression of *Pdk1:Akt* and *Ask1*^{K618M}. White lines in H indicate areas of *ptc*>expression. TP3 is used to stain nuclei. Scale bars: 50 µm.

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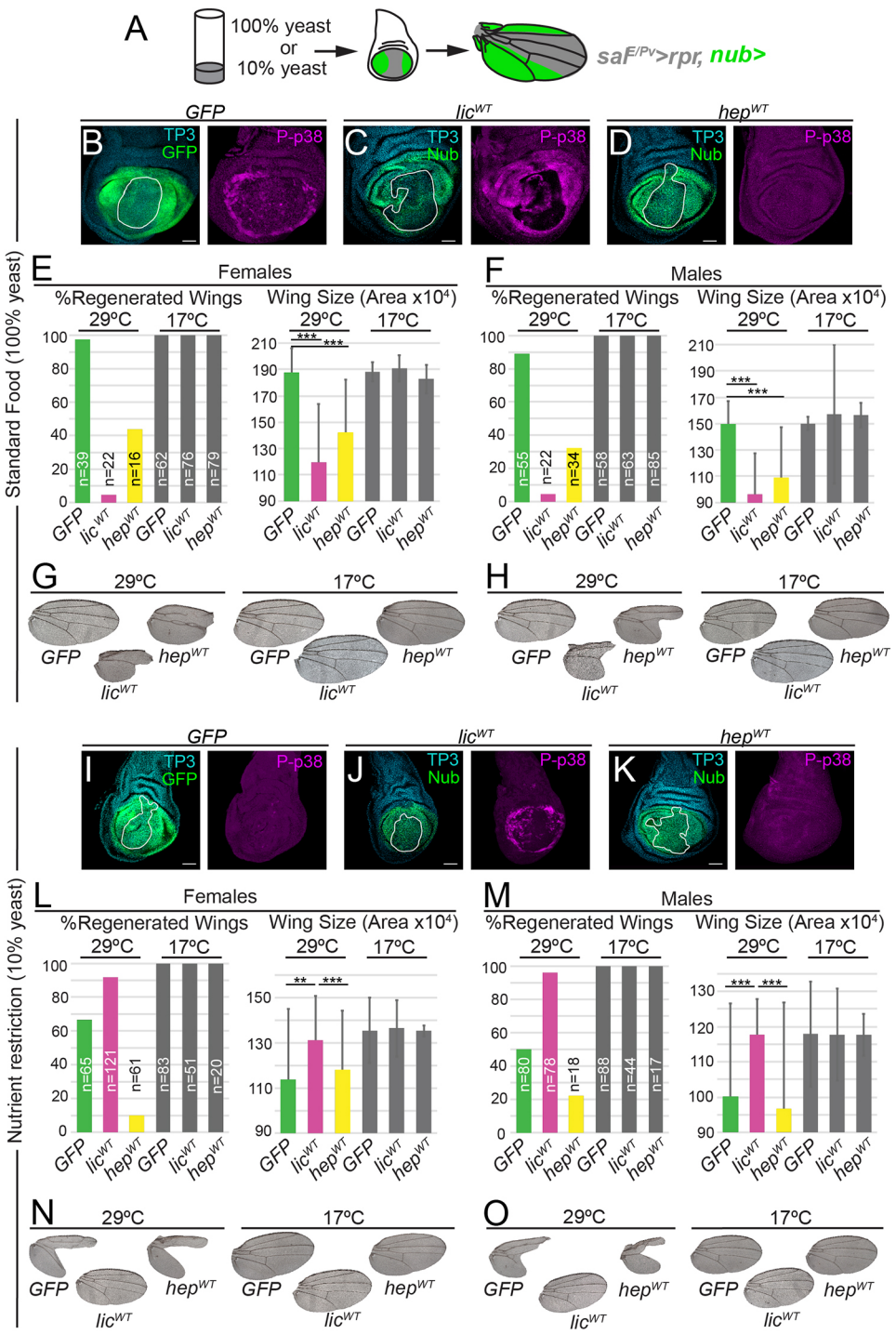

Fig. 3. The MAPKK *licorne* (*lic*^{WT}), but not *hemipterous* (*hep*^{WT}), reverses the failure in regeneration caused by nutrient restriction. (A) Zones of dual transactivation. Gray, apoptotic zone generated by *sal*$^{E/Pv}$-*LHG LexO-rpr* (*sal*$^{E/Pv}$*>rpr*); green, transgene activation under *nub-Gal4* (*nub>*) to express the indicated UAS-transgene. (B-D) P-p38 of *nub>GFP* (B), *nub>lic*^{WT} (C) and *nub>hep*^{WT} (D) discs in standard food conditions. (E,F) Percentage of regenerated wings and wing areas in females (E) and males (F). (G,H) Examples of wings of the indicated genotypes and at indicated temperatures. (I-K) P-p38 of *nub>GFP* (I), *nub>lic*^{WT} (J) and *nub>hep*^{WT} (K) in nutrient restriction conditions. (L,M) Percentage of regenerated wings and wing size in females (L) and males (M). (N,O) Examples of wings of the indicated genotypes and at indicated temperatures. ***P*<0.01, ****P*<0.001. White lines in the confocal images outline pyknotic nuclei of apoptotic cells. TP3 is used to stain nuclei. Scale bars: 50 µm.

nutritional status. In control animals (10% yeast) kept at 17°C to block cell death, the wings were normal, but smaller (Fig. 3N,O).

Next, we analyzed whether ectopic activation of p38 could restore regeneration in nutrient restricted animals. As constitutive activation of p38 can cause cell death (Huang et al., 2016; Sun et al., 2019), we used a wild-type allele of *licorne* (*lic*^{WT}), a serine/threonine kinase that phosphorylates p38. This resulted in moderate levels of P-p38 and a few scattered apoptotic cells (Fig. S2G). Expression of *lic*^{WT} under normal food conditions did not allow regeneration (Fig. 3E, F), likely because of the toxicity resulting from the excessive levels of P-p38 caused by both ectopic expression and genetic ablation (Fig. 3C). However, following nutrient restriction, the levels and distribution of P-p38 were similar to those of GFP in the animals grown in standard food conditions (compare Fig. 3J with 3B). The percentage of regenerated wings in *lic*^{WT}-expressing flies increased above 90% (Fig. 3L,M). In addition, the wing size increased to levels that were similar to those of the controls at 17°C.

We also tested whether JNK could restore regeneration. JNK signaling was induced moderately by the expression of the wild-type form of the *Drosophila* JNKK, *hemipterous* (*hep*^{WT}) (Uhlirova and Bohmann, 2006). The expression of *hep*^{WT}, but not of *GFP* or *lic*^{WT}, resulted in an increase of P-JNK (Fig. S4A-G). The activation of *hep*^{WT} did not result in P-p38 around the apoptotic zone of the discs (Fig. 3C,J) and did not reverse the defects in regeneration

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resulting from nutrient restriction (Fig. 3E,F,L,M). Altogether, these results showed that lic^{WT}, but not hep^{WT}, was able to revert the effects of nutrient limitation, indicating that the p38 pathway is highly sensitive and responsive to nutrients to drive regeneration.

To further demonstrate that p38 activation was driven through the Ser83 residue of Ask1, we combined cell death with the expression of the $Ask1^{S83A}$ mutant (Fig. 4A). Under these conditions, P-p38 levels were no higher than basal levels (Fig. 4B), while wing regeneration dropped to around 10%, the wing size was reduced (Fig. 4E) and wings showed patterning defects (Fig. 4G,H). Concomitant expression of $Ask1^{S83A}$ and lic^{WT} resulted in higher levels of P-p38 (Fig. 4C), and an increase in the percentage of regenerated wings, to up to more than 67%, as well as an increase in the wing size (Fig. 4E,F) and recovering of the wing pattern (Fig. 4G,H). By contrast, P-p38 staining did not change after the ectopic expression of $Ask1^{S83A}$ and hep^{WT} (Fig. 4D), and did not lead to recoveries in the percentages of regenerated wings, wing size or patterning (Fig. 4E-H). In these $Ask1^{S83A}$ experiments, the expression of hep^{WT}, but not of lic^{WT} or *GFP*, caused the phosphorylation of JNK in the *nub*>domain (Fig. S4H-K).

From our results, we concluded that Ser83 of the Ask1 YH_GVRESF sequence is necessary for integrating nutrient signals and the insulin pathway to initiate the p38-dependent damage response. It has been demonstrated that Ask1 and p38 play an important role in the stress-triggered regeneration of the gut (Patel et al., 2019). Perhaps not only in discs, but also in other organs such as the gut, the synergy between ROS and nutrients/Akt through the Ser83 residue of Ask1 is necessary for the activation of p38.

p38 and JNK respond to damage differently. It is known that JNK is associated with cell death and that there is a mutual antagonism between p38 and JNK (Wagner and Nebreda, 2009). Dying cells lack P-p38, possibly because of a reduction of P-Akt levels in apoptotic cells (Franke et al., 2003), and therefore they are unable to attenuate P-Thr Ask1 activity. Indeed, P-Thr Ask1 has been found to be highly accumulated in the apoptotic zones of the disc (Santabárbara-Ruiz et al., 2019). We speculate that this could result in the activation of JNK in damaged cells, which then promotes apoptosis (Shlevkov and Morata, 2012). Although P-JNK is not abundant in dying cells, reporters of JNK signaling have been associated with apoptotic cells (Pinal et al., 2019). As previously suggested, the attenuated form of Ask1 might not only be key for p38 activation, but also for maintaining low levels of JNK in regenerating cells (Santabárbara-Ruiz et al., 2019). Therefore, both MAPKs play a role in regeneration: p38 in a manner that is dependent on nutrients and on PI3K/Akt; and JNK independently of them. In addition to the attenuated or low levels of Ask1, the MAP3 kinase Tak1 could operate independently of nutrients to activate JNK (La Marca and Richardson, 2020).

In summary, we propose two roles for Ask1 in regenerating cells: one in the attenuation of catalytic activity to avoid cell death in regenerating cells; and the other in the activation of p38 to propel regeneration. In the future, it will be fundamental to unveil the mechanisms of tissue repair triggered by p38.

MATERIAL AND METHODS

Drosophila strains

The $sal^{E/Pv}$-*LHG*, *lexO-rpr*, *UAS-*$Ask1^{WT}$ and *UAS-*$Ask1^{S83A}$ *Drosophila melanogaster* strains have been previously described (Santabárbara-Ruiz et al., 2015; Santabárbara-Ruiz et al., 2019), as have *UAS-*$lic^{WT1.1}$

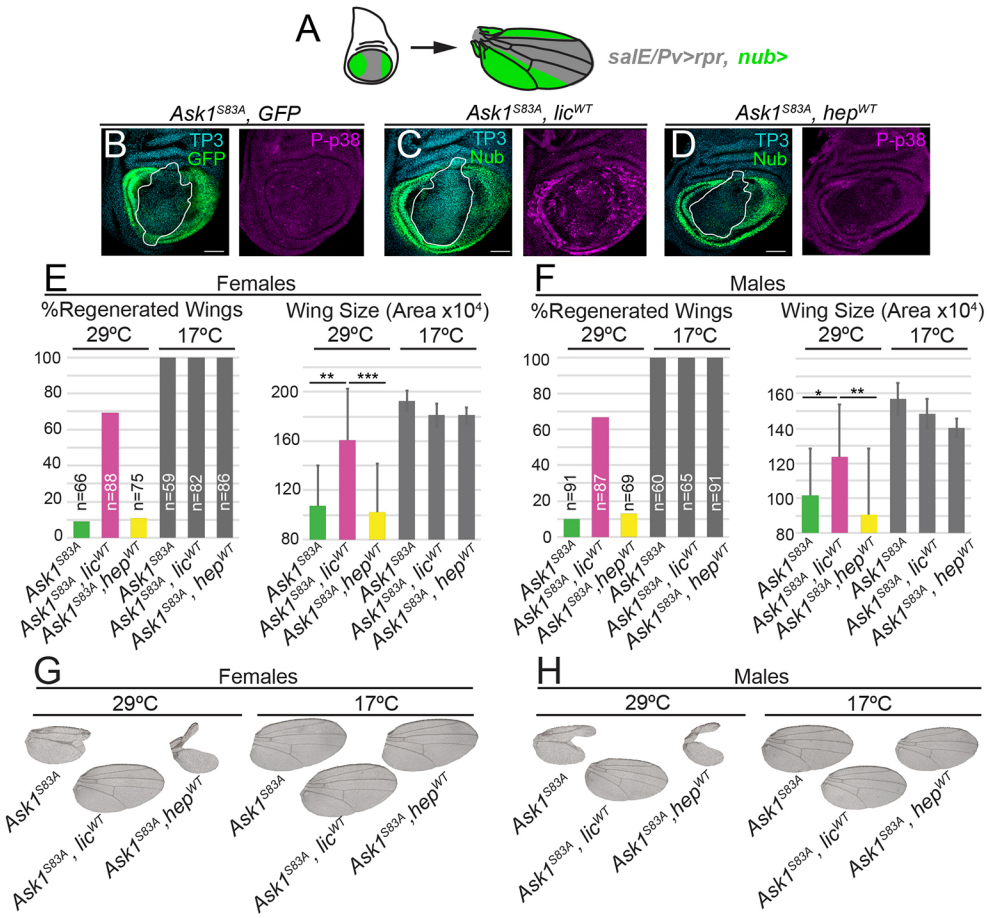

Fig. 4. The MAPKK *licorne* (lic^{WT}), but not *hemipterous* (hep^{WT}), reverses the failure in regeneration caused by Ask^{S83A} expression. (A) Zones of dual transactivation. Gray, apoptotic zone ($sal^{E/Pv}$>*rpr*); green, *nub*> zone of UAS-transgene expression. (B-D) P-p38 of *nub*>$Ask1^{S83A}$, *GFP* (B), *nub*>$Ask1^{S83A}$, lic^{WT} (C) and *nub*>$Ask1^{S83A}$, hep^{WT} (D) wing discs. (E,F) Percentage of regenerated wings and wing areas in females (E) and males (F). *P<0.05, **P<0.01, ***P<0.001. (G,H) Examples of wings. White lines in the confocal images outline pyknotic nuclei of apoptotic cells. TP3 is used to stain nuclei. Scale bars: 50 µm.

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(Terriente-Félix et al., 2017), *UAS-hep*^{WT} (Uhlirova and Bohmann, 2006), *UAS-Ask1*^{K618M} (Kuranaga et al., 2002), *UAS-Pdk1* and *UAS-Pkb1/Akt1* (which were recombined and a gift from H. Stocker, ETH, Zürich, Switzerland), and *sal*$^{E/Pv}$*-Gal4* (Santabárbara-Ruiz et al., 2015). The following strains were provided by the Bloomington Drosophila Stock Center: *tubGal80*^{TS} (RRID:BDSC_7017), *ptc-Gal4* (RRID:BDSC_2017), *UAS-GFP* (RRID:BDSC_4776) and *UAS-Ask1*^{RNAi} (RRID:BDSC_35331). The following are described in FlyBase: *hh-Gal4, dpp-Gal4* and *nub-Gal4*. The *UAS-bsk*^{RNAi} strain was obtained from the Vienna Drosophila Resource Center (VDRC V104569).

The Gal4/UAS/Gal80^{TS} system for transgene activation in the wing imaginal disc

The *ptc-Gal4* strain expressed Gal4 in a central stripe of the wing imaginal discs. Experiments with the *UAS-Ask1*^{K618M} mutant were also performed with *dpp-Gal4*. Both the *ptc-Gal4* and *dpp-Gal4* drivers show a similar pattern of expression in a stripe of cells. The controls carried *ptc-Gal4 UAS-GFP (ptc>GFP)*, whereas the other genotypes were stained with the anti-Ptc antibody to identify *ptc* zone.

A recombinant of two UAS lines was used to activate the PI3K/Akt pathway – *UAS-Pdk1:UAS-Akt1 (Pdk1:Akt* in the text and figures). The expression of *UAS-Pdk1:UAS-Akt1* and the other transgenes was controlled by the thermo-sensitive Gal4 repressor *tubGal80*^{TS}.

Drosophila crosses were cultured to lay eggs for 24 h at 17°C. Embryos were kept at 17°C until the 7th day (168 h) after egg laying to prevent *UAS-Pdk1:UAS-Akt1* expression. Larvae were subsequently moved to 29°C for 24 h and then the imaginal discs from wandering larvae were dissected and processed for staining and immunofluorescence studies.

Immunofluorescence and the TUNEL assay

Immunostainings were performed using standard protocols. The primary antibodies used in this study were: the cleaved *Drosophila* Dcp1 antibody (Cell Signaling, 9578S; 1:200), anti-Ptc antibody (DSHB; 1:100), anti-P-p38 antibody (Cell Signaling, 9211S; 1:50), anti-P-Akt antibody (S473, Cell Signaling; 1:100), the rabbit anti-ACTIVE JNK pAb (V7931, Promega; 1:100), anti-MMP1 antibody (DSHB, 14A3D; 1:200) and anti-nubbin antibody (1:50; from S. Cohen, University of Copenhagen, Denmark). The anti-ACTIVE JNK pAb antibody (anti-P-JNK) also labels mitoses as spindle pole proteins associate with JNK (Lim et al., 2015), and responds to the ectopic activation of the wild-type form of the JNKK *licorne* (*UAS-lic*^{WT}) and a dominant-negative form of the JNK *basket* (*UAS-bsk*^{DN}) (Fig. S2). The anti-phospho-Ask1(Thr845) polyclonal antibody (Cell Signaling, 3765; 1:200) was also used, which labels residues surrounding Thr845 of mouse ASK1 only when Thr845 is phosphorylated. This antibody (P-Thr in Fig. S1) was used to monitor Ask1 activity.

Fluorescently labeled secondary antibodies were from Thermo Fisher Scientific. Nucleic acid staining was performed by incubating discs for 10 min with 1 µM TO-PRO-3 (TP-3) or 10 µg/ml of DAPI (Life Technologies). The discs were mounted in SlowFade (Thermo Fisher Scientific).

For the detection of apoptotic cells, we also used the TUNEL assay. We employed the fluorescently labeled Alexa Fluor 647-aha-dUTP (Thermo Fisher Scientific), incorporated using terminal deoxynucleotidyl transferase (Roche). Apoptosis was also detected after cleaved *Drosophila* Dcp1 antibody immunofluorescence.

Image acquisition

For the confocal images, a Zeiss LSM880 and a Leica SPE confocal laser scanning microscopes were used. Images were analyzed using FIJI. Data on mean pixel intensity graphics were collected from the *ptc* zone. A Leica DMLB microscope was used for taking pictures of the adult wings.

Genetic ablation and the dual Gal4/LexA transactivation system

For adult wing regeneration analysis, we used a dual Gal4/LexA transactivation system. We used the *sal*$^{E/Pv}$*-LHG* and *LexO-rpr* strains for genetic ablation, using the same design as that for Gal4/UAS. The LHG is a modified version of lexA that contains the activation domain of Gal4 separated with a hinge construct. This form is suppressible by *tubGal80*^{TS} (Yagi et al., 2010). The Gal-4 line used was *nubbin-Gal4* (*nub>*), which is expressed in the entire wing pouch. *UAS-GFP* (GFP) on an antibody against Nubbin (Nub) were used to monitor the *nub>*zone.

Animals laid eggs for 6 h at 17°C. Embryos were kept at 17°C until day 8 (192 h after egg laying) to prevent *rpr* expression. They were subsequently moved to 29°C for 11 h and then back to 17°C to allow the tissue to regenerate. Two types of controls were used in parallel for each genotype: (1) individuals carrying *UAS-GFP* as the neutral transgene, which were moved to 29°C for 11 h, as in the experimental condition; and (2) individuals kept continuously at 17°C to avoid any transgene activation to control for possible effects of transgene insertion. Colored bars in histograms correspond to the experiments of cell death induction and transgene expression conducted at 29°C. Gray bars correspond to the control experiments performed at 17°C without cell death induction or transgene expression. From the experiments performed at 29°C, a representative anomalous wing phenotype is shown in the corresponding figures. In addition we scored wing pattern and size of the different transgenes expressed under the *nub-Gal4* in unablated discs.

Nutrient restriction conditions

One liter of *Drosophila* medium contained 64 g of fresh yeast, 67.2 g of dextrose, 40 g of wheat flour and 8.8 g of Bacto agar, here referred to as standard medium (or 100% yeast food). Food for nutrient restriction (or 10% yeast food) was made by reducing the amount of yeast (6.4 g/l) without altering the other ingredients. All crosses and experiments were performed under non-crowding conditions.

Nutrient restriction experiments were performed as follows. Embryos were cultured at 17°C. On day 7 (168 h) after egg laying, the larvae were removed from their standard food conditions, washed in PBS and transferred to a tube with only 10% yeast. In the control animals, the same procedure was followed, except that they were transferred to another tube containing standard food. On day 8 (192 h) after egg laying, the larvae were then moved to 29°C for 11 h to activate the whole set of transgenes as the inhibitor Gal80 is inactive at this temperature. After that, the larvae were transferred to 17°C to allow the tissue to regenerate. Thus, from 24 h before cell death induction, during the cell ablation period and during the entire regeneration process up to adulthood, the larvae were in conditions of nutrient restriction.

To exclude any toxicity due to the insertion of the transgene, all experiments were carried out in parallel, but constantly at 17°C to maintain *tubGal80*^{TS} activity and block transgene (UAS- or lexO-) expression. Under these conditions, no defects in patterning were detected. However, all the flies raised under starvation conditions had smaller wings compared with flies reared on normal food.

Statistical analysis

To test the capacity to regenerate in different genetic backgrounds, we examined adult wings obtained from *sal*$^{E/Pv}$*>rpr* individuals in which patterning and size defects can be scored easily. Flies were fixed in glycerol:ethanol (1:2) for 24 h. The wings were dissected in water and then washed with ethanol. Subsequently, they were mounted in lactic acid:ethanol (6:5), and analyzed and imaged under a microscope.

The percentage of regenerated wings refers to fully regenerated (for genetic ablation genotypes) or normally developed wings (for testing transgenes), and was calculated according to the number of wings present with a complete set of veins and interveins as markers of normal patterning. Wing areas were measured as an indication of wing size. Areas of the mounted wings were outlined and scored using FIJI.

In all the figures (wing areas and mean pixel intensity graphics), data are mean±s.e.m.. To make statistical comparisons, we used one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test to make pair comparisons between each group using IBM SPSS Statistics. Significance is indicated in the figures only when $P<0.05$, as follows: *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Acknowledgements

We thank Hugo Stocker, Mar Fernández, Paula Santa Bárbara, Carlos Camilleri and Paula Climent for their comments. We thank Manel Bosch from the CCiT-UB imaging facilities for support and Concepcion Arenas for advice on statistical analysis.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: M.C., F.S.; Methodology: J.E.; Validation: J.E.; Formal analysis: J.E., M.C., F.S.; Investigation: J.E., F.S.; Data curation: J.E.; Writing - original draft: F.S.; Writing - review & editing: J.E., M.C., F.S.; Supervision: F.S.; Project administration: M.C., F.S.; Funding acquisition: M.C., F.S.

Funding
This research was funded by the Spanish Ministerio de Ciencia, Innovacion y Universidades (PGC2018-099763-B-I00 to F.S. and M.C.), by the Institució Catalana de Recerca i Estudis Avançats (ICREA Academia award to M.C.) and by the Agència de Gestió d'Ajuts Universitaris i de Recerca (2017SGR1455 to F.S. and M.C.).

Peer review history
The peer review history is available online at https://journals.biologists.com/dev/article-lookup/doi/10.1242/dev.197087

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