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Study of early signaling events governing epithelial regeneration

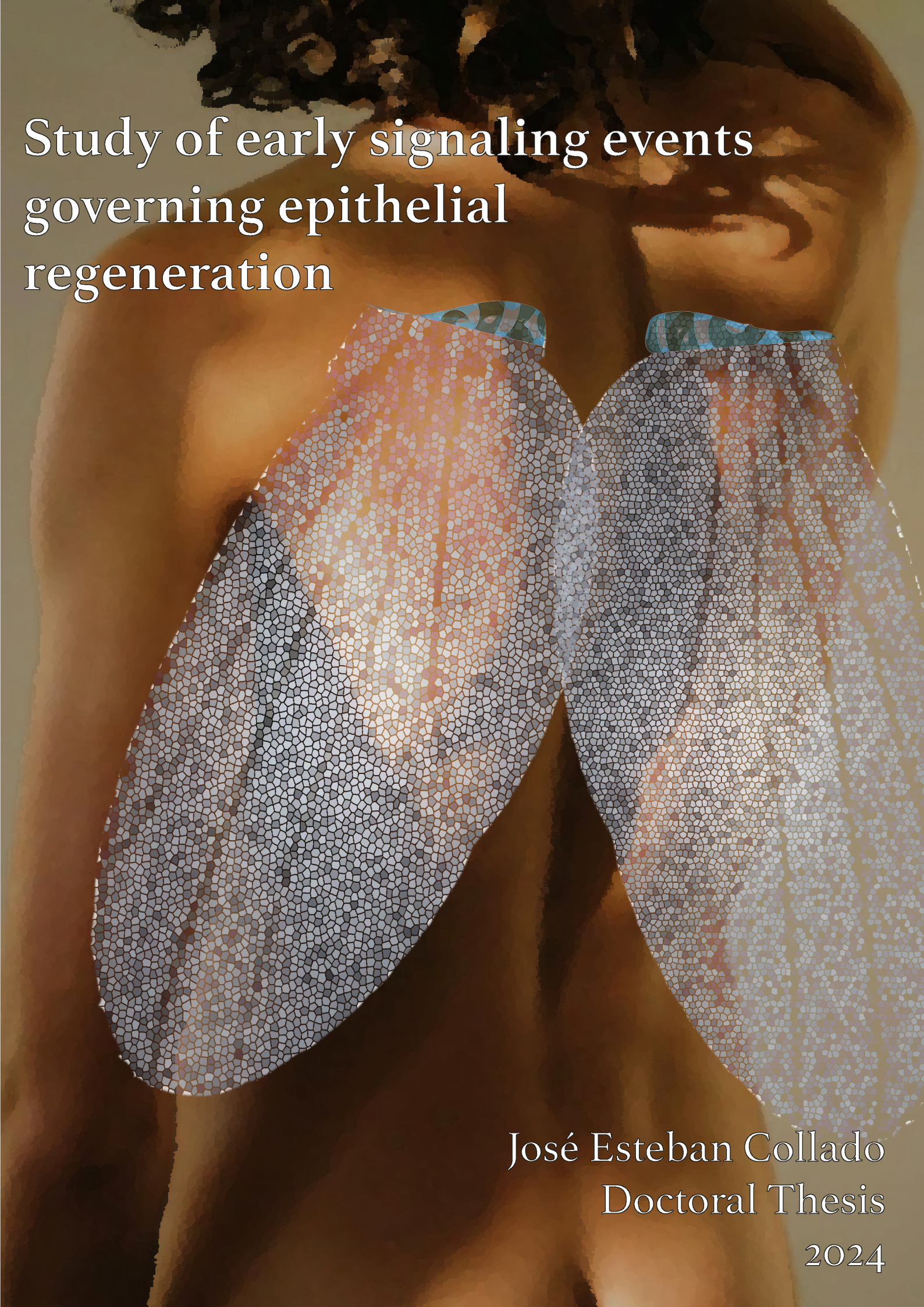
Estudio de las señales tempranas que dirigen
la regeneración de epitelios

José Esteban Collado

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The background of the cover is a composite image. The upper portion shows a close-up of a person's face, focusing on the eyes and nose, with a warm, golden-brown color palette. The lower portion shows two hands holding a model of a developing embryo, which is covered in a fine, mosaic-like pattern of small, irregular shapes in shades of blue, green, and yellow. The overall composition suggests a connection between human biology and developmental science.

Study of early signaling events governing epithelial regeneration

José Esteban Collado
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Study of early signaling events governing epithelial regeneration

(Estudio de las señales tempranas que dirigen la
regeneración de epitelios)

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Front cover explanation.

A modern Gregor Samsa, naked, with his body covered by two epithelia, the human skin (still) and two wings (also epithelial tissue) of *Drosophila* that emerge from his shoulder blades and cover his back. How long will it take for this young, beautiful, and intelligent man to complete his *metamorphosis*? How much time does he have left until all those who loved him end up repudiating and forgetting him?

Explicación de la portada.

Un moderno Gregorio Samsa, desnudo, con su cuerpo cubierto por dos epitelios, la piel humana (todavía) y dos alas (también tejido epitelial) de *Drosophila* que nacen de sus omoplatos y le recubren la espalda. ¿Cuánto tardará este hombre joven, bello e inteligente en terminar su *metamorfosis*? ¿Cuánto tiempo le queda hasta que todos los que le amaron lo acaben repudiando y olvidando?

Back cover explanation.

Among the black walls of a forgotten temple, light makes its way, the same light Prometheus stole from the Gods of Olympus, shining through a stained glass window. This stained glass window, which in many temples illustrates myths and cults, is in this case a fragment of the imaginal wing disc of *Drosophila*. The lilac line running through the center represents the *ptc* region dying under the expression of a pro-apoptotic gene. The blue glasses represents the cell divisions that will restore the lost tissue.

Explicación de la contraportada.

Entre los negros muros de un templo olvidado se abre paso la luz, esa que robó a los Dioses del olimpo Prometeo, a través de una vidriera. La vidriera, que en muchos templos ilustra mitos y cultos, es en este caso un fragmento del disco imaginal de ala de *Drosophila*. La línea lila que recorre el centro es la región de *ptc* muriendo bajo la expresión de un gen pro-apoptótico. Los vidrios azules, las divisiones celulares que recompondrán el tejido perdido.

*“Antes la **muerte** que la **salud** que se nos propone”*

Gilles Deleuze, Lógica del sentido, 1969

*“En este país, en general, no se paga el trabajo, sino la **sumisión**”.*

Pío Baroja, El árbol de la ciencia, 1911

Dedicatoria

Escribo esta dedicatoria mientras acabo de maquetar y perfilar la presente tesis, al borde del agotamiento mental. Es en esos momentos; cuando se está sufriendo, como decía mi abuela, cuando uno distingue realmente lo importante de lo efímero y accesorio.

A Florenci, perquè hem parlat, raonant, argumentat i, fins i tot discutit, però el resultat ha estat sempre l'equilibri. I més enllà, la sensació d'haver estat treballant braç a braç per dur a terme una feina sempre **rigorosa**, que, amb els temps que campen, ja és molt a dir i una cosa per la em sento orgullós i agraït. També a Montse i a Marta, per què m'han estat portant pel camí de la ciència: La Montse amb la seva crítica directa i feroç (que ha contribuït molt en això la feina **rigorosa**). I a la Marta per ser el màxim exponent del dubte, per veure les esquerdes allà on ningú més les veuria. També per la seva sinceritat, què és molt d'agrair en aquest *field* on molts cops està amagada.

A mis amig@s, pero, por encima de todo, a los que he encontrado aquí en Barcelona. Por haber sido sucesión primaria que ha convertido este yermo páramo en un nuevo **hogar**. Por abrir mis horizontes, por darme consuelo y sosiego. Sois el barco que me mantiene a flote en el mar de los últimos 7 años. A flote y lejos de la **soledad** que condena las almas.

A mis abuelas, que sin yo saberlo (no por no escuchar, sino por estar aún dentro de la crisálida de la infancia) fueron las primeras en hablarme de (casi) todo.

A mi padre. Porque **soy** su imagen especular distorsionada sobre la superficie de un arroyo.

A mi madre. Sin paliativos, la mejor persona (o la más buena) que conozco. Por ser la **estrella** al final de todos mis caminos, la luz que sigue brillando cuando todo parece apagarse en la fría noche.

A mi hermana. Por ser mi puerto y, a la vez, mi viento. La única persona a la que confiaría (y confío) enteramente mi **alma**.

JEC
2024

Abstract

The mechanisms through which cells communicate to initiate a regenerative response remains unsolved. It is established that Reactive Oxygen Species (ROS) are early signals released from injured cells that propagates to stimulate the replacement of lost tissue. ROS triggers the activation of the stress activated protein kinases (SAPKs) p38 and JNK via phosphorylation. Prolonged or elevated activation of these kinases may induce apoptosis, while brief or minimal activation can foster regeneration. Nonetheless, the precise connection between ROS generation and the initiation of regenerative signaling pathways remains incompletely elucidated. To this aim, we used *Drosophila* wing imaginal discs as a model system due to its well-characterized regenerative ability after injury or genetic ablation. We found that The Apoptosis signal-regulating kinase 1 (Ask1) emerges as a pivotal factor for driving regenerative growth. It exhibits the remarkable ability to sense ROS both in dying and living cells. However, in living cells, its activation is intricately regulated by nutrient sensitivity through Akt, a core kinase of the insulin (PI3K/Akt) pathway. Akt phosphorylates Ask1 at Ser83, an indispensable event for Ask1 to catalyze the activation of p38, albeit not JNK. Moreover, nutrient restriction or mutations targeting Ser83 of the *Drosophila* Ask1 block regeneration. Impediments that can be rescued by the ectopic activation of p38, but not JNK. Besides, ROS plays a critical role in the ligand-independent TNF α receptor (TNFR) Wengen (Wgn) activation in response to apoptosis. Wgn, but not Grindelwald (Grnd), exhibits a protective mechanism mediated by the signaling molecule TRAF1, ultimately leading to the activation of the Ask1-p38 axis. This stress-response mechanism could be evolutionary conserved as Wgn cysteine rich domain (CRD) shows greater similarity with TNFR families found in other deuterostomes. Our findings underscore a non-autonomous activation of a ROS sensing mechanism orchestrated by Ask1, Akt1 and Wgn that results in the activation of a p38-dependent regenerative response.

Resumen

Los mecanismos celulares de comunicación en el inicio de la respuesta regenerativa están todavía sin resolver. Las Especies Reactivas del Oxígeno (ROS) son señales tempranas liberadas por las células lesionadas que se propagan para estimular la reparación del tejido dañado. ROS desencadena la activación de las proteínas quinasas activadas por estrés (SAPKs); p38 y JNK, mediante la fosforilación. La activación prolongada o elevada de estas quinasas puede inducir apoptosis, mientras que una breve o mínima promueve la respuesta regenerativa. Sin embargo, la conexión precisa entre la generación de ROS y la iniciación de estas vías de señalización no está completamente dilucidada. Con el fin de resolver esta cuestión, utilizamos los discos imaginales de ala de *Drosophila* como sistema modelo, debido a su contrastada capacidad regenerativa tras sufrir lesiones físicas o ablación genética. Encontramos que la Quinasa Reguladora de la Señal de Apoptosis 1 (Ask1) es un factor crucial para impulsar el crecimiento regenerativo. Ask1 detecta la presencia de ROS tanto en células muertas como vivas. Sin embargo, en las células vivas, su activación está claramente regulada por la sensibilidad a los nutrientes a través de Akt, una quinasa central de la vía de insulina (PI3K/Akt). Akt fosforila Ask1 en el residuo Ser83, un evento indispensable para que Ask1 catalice la activación de p38, pero no de JNK. Además, la restricción de nutrientes o mutaciones que afectan a la Ser83 de Ask1 de *Drosophila* bloquean la regeneración. Obstáculos que pueden ser rescatados por la activación ectópica de p38, pero no de JNK. ROS también juega un papel crítico en la activación del receptor de factor de necrosis tumoral α (TNFR) Wengen (Wgn) en respuesta a la apoptosis de forma independiente al ligando. Wgn, pero no Grindelwald (Grnd), exhibe un mecanismo protector mediado por la molécula señalizadora TRAF1, lo que conduce finalmente a la activación del eje Ask1-p38. Este mecanismo de respuesta al estrés podría estar conservado evolutivamente, ya que el dominio rico en cisteínas (CRD) de Wgn muestra una mayor similitud con las familias de TNFR encontradas en otros deuterostomados. Nuestros hallazgos revelan una activación no autónoma de un mecanismo de detección de ROS orquestado por Ask1, Akt1 y Wgn que resulta en la activación de una respuesta regenerativa dependiente de p38.

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General Introduction

1. Regeneration

1.1 From myth...

A legend is a symbolic tale, often with an unclear origin and rooted in tradition, ostensibly recounting real occurrences, particularly linked with religious convictions. It stands apart from symbolic practices (like cults or rituals) and symbolic sites or items (such as icons or temples) (Buxton, Bolle and Smith, 2024). The tale of Osiris, among the earliest documented instances mentioning the idea of regeneration in ancient Egypt, is etched on a chamber wall within Teti's pyramid (David, 2002). Osiris was slain by Seth, who dismembered the corpse into 14 fragments scattered across Egypt. Ultimately, Isis and her sister Nephthys located and interred all the pieces, save for the phallus. The fragments reunited and regenerated, restoring Osiris to a new existence, albeit now as the ruler and arbiter of the underworld (Assmann and Lorton, 2001). Fifteen centuries later, the renowned Greek poet Hesiod presented the earliest written account of the Prometheus Myth in the late 8th century BC, within his work 'Theogony.' Prometheus is occasionally identified as the deity of Fire. He pilfered fire from the Olympian deities and bestowed it upon humanity in the guise of knowledge and technology. As punishment for stealing fire from Olympus, Zeus, the king of the Olympian gods, condemned Prometheus to endure perpetual torment: Prometheus was tethered to a rock in the Caucasus Mountains, while an eagle —the symbol of Zeus— devoured his liver (in ancient Greece, the liver was believed to house human emotions). His liver would then grow back overnight, only to be eaten again the next day in an ongoing cycle (Weinfield, 2006). But, did the ancient Greeks already have knowledge of the liver's ability to regenerate and of the fact that this capacity is inexhaustible? (Power and Rasko, 2008).

If so, we don't have any written record left about this knowledge from that time, and we have to end this story, as Hesiod did, with the freed of Prometheus from its torment by the hero Heracles. By chance (or not) it is the same hero, Heracles, the main character of another Greek Myth: The myth of Hydra, a serpentine water monster which lair was the lake of Lerna (the entrance for the Underworld in Greek mythology) (C.Kerényi, 1978). According to Hesiod the monster is killed by Heracles as the second of his Twelve Labors. The Hydra possessed many heads, and later versions of the Hydra story add a regeneration feature to the monster: for every head chopped off, the Hydra would regrow two heads (Ogden, 2013). Heracles required the assistance of his nephew Lolaus to cut off all of the monster's heads and burn the neck using a sword and fire (Weinfield, 2006).

1.2 ...To science: Early studies on regeneration, From Trembley to Morgan

Twenty-six centuries after Hesiod, the first scientific experimental studies on regeneration were published by the Swiss naturalist Abraham Trembley (Vartanian, 1950; Galliot, 2012a, 2012b). Trembley discovered that the heads of Hydra (name given to his mysterious, sometimes multi-headed, regenerating polyps by Trembley inspired by the myth) and earthworms' regrow after been removed (Lenhoff and Lenhoff, 1989). However, the questions of why and how this process occurs remained unsolved at that time.

General Introduction

At the turn of the twentieth century, it had already been established that many organisms were capable of regeneration. Concomitantly three models for the understanding of development were proposed with implications in the comprehension of regeneration:

1. The German zoologist Wilhelm Roux discovered that if he killed one of the first-formed blastomeres in frog embryos, the surviving one would develop into a half-embryo. He interpreted this as evidence that each cell developed independently (Sander, 1997). Consequently, development was understood as a summation of multiple mosaic developments.
2. It was due to a “vital force”: Hans Driesch, contemporary of Roux, challenged his observations. He dissected early-stage sea urchin embryos and showed that new embryos grew from the cells he had removed from the original ones. Driesch interpreted that it was due to a “vital force” or, in more current terms, a regulatory capacity of development. Driesch emphasized epigenetic, external factors of development, as opposed to the internal focus of Roux (Khaner, 1993).
3. It was an adaptative effect. August Weismann, a German evolutionary biologist, give some conclusions about regeneration: He postulated that regenerative capacity is an adaptive trait that is subject to phylogenetic alterations and therefore may vary considerably among the taxa [(Weismann, 1893) and reviewed in (Elchaninov, Sukhikh and Fatkhudinov, 2021)]. He claimed that germ cells resided throughout the body or in those parts liable to injury (like the claws of a crab or the tentacles of a hydra). He also predicted that these germ cells, would be unique depending on their location in different body layers (Weismann, 1834-1914). Weismann identified regeneration as a different process from development, an assumption that distinguished his theoretical framework.

With these three interpretations in mind, the American biologist T.H. Morgan started his studies about regeneration in the early 20th century. Although best known as a pioneer in genetics of the fruit fly *Drosophila*, Morgan’s first passion was development and regeneration. T.H. Morgan studied regeneration in earthworms, planarians and hydra (Sunderland, 2010). In his book *Regeneration* (1901), Morgan made several contributions to the knowledge of regeneration biology: (1) adopting a clear and consistent terminology, (2) propose that the regeneration was, in general, a developmental characteristic. This allowed him to justify inferences between regeneration and development and ultimately argue that a comprehensive theory of development needed to account for regeneration. (3) The tensions hypothesis: a mechanistic theory that offered a causal explanation for regeneration involving internal factors. His theory account for regulation within the organism, in particular the concept of totipotency: the ability of a cell to travel down different developmental trajectories in different contexts.

But, as many other seminal studies the work of Morgan left many questions unsolved: What causes differentiation? How are developmental processes coordinated? How can organs regenerate in the absence of developmental cues?

1.3 A current definition of regeneration

Morgan's definition of regeneration stills being correct nowadays. But we can try to give a current definition, taking into account the latest studies in the field: Regeneration is the ability of an organism to reconstruct body parts that have been lost or damaged after trauma, restoring again complete structure and function. Also, replacement or normal turnover of some tissues under homeostasis conditions is also called regeneration by some authors.

1.4 Types of regeneration

Classically, regeneration has been classified as epimorphic or morpholactic according to whether a blastema is formed or not after wound healing (see Sunderland, 2010 for revision).

Epimorphosis occurs, for example, in planarian and amphibian limb regeneration. A blastema is formed at the site of the wound and is formed of undifferentiated cells. Eventually, this blastema regenerates the lost tissue in a "add on" process of newly generated cells (reviewed in Baguñà, 2019).

In contrast, Hydra regeneration happens without the formation of blastema. A rearrangement of pre-existing cells in the stump occurs. This type of regeneration based on tissue remodelling is called morphallaxis (Chera *et al.*, 2009; Galliot and Chera, 2010; Galliot, 2011).

However, some observations challenge this classification. In urodele limb regeneration, the distal portion of the body is formed immediately, and the interaction of this distal part with the remaining proximal portion may induce reorganization of positional information. Subsequently, lost tissue is restored by intercalation (Nye *et al.*, 2003). Given that certain regeneration events cannot be neatly classified into either model (epimorphosis or morpholaxis), some authors suggest more useful terms such as "distalization" and "intercalation" to explain the principles of regeneration (Agata, Saito, and Nakajima, 2007).

1.5 Biological levels of regeneration

It has been proposed that regeneration can occur at five levels of biological organization across metazoans (Bely and Nyberg, 2010; Slack, 2017). Whole-body regeneration as planarians and hydra which can reconstruct an entire body when cut in small fragments. Structural regeneration occurs in some arthropods and vertebrates that have the ability to reconstruct appendages. The size restoration of an organ is called organ regeneration, as it occurs in the human liver after partial hepatectomy. Tissue regeneration is considered as the restoration of homeostasis in a given homogeneous cell population, for example recovery of skin epithelium after damage. And finally, cellular regeneration refers to recovery of a damaged cell, as in nerve axon regrowth. After injury dramatic changes at cellular level are required. Regenerating tissues initiate a complex program including diverse processes such as cell death, wound healing, dedifferentiation, and stem cell and progenitor cell proliferation. Also, regenerated tissues should integrate

polarity and positional identity cues with pre-existing body structures to restore the original shape and form of the lost structure, (King and Newmark, 2012).

1.6 Evolution of regeneration in metazoans

The variation in regeneration capacity among metazoans (Tanaka and Reddien, 2011; Vríz, Reiter and Galliot, 2014; Chen and Poss, 2017; Reddy, Gungi and Unni, 2019) can be attributed to several factors, including phylogenetic distribution and the interplay of various evolutionary, ecological, and developmental processes, reviewed in (Alvarado and Tsonis, 2006). Addressing different points, is crucial for understanding this variation:

1. **Phylogenetic Distribution:** Regeneration ability varies across different taxonomic groups. Some taxa, such as planarians, hydra, and certain species of salamanders, exhibit remarkable regenerative abilities, while others, like mammals, have limited regenerative capacities. Understanding the phylogenetic distribution of regeneration can provide insights into the evolutionary history of this trait and the selective pressures that have shaped its diversity (Bely and Nyberg, 2010).
2. **Evolutionary Origins:** The origin of regeneration and its subsequent maintenance or loss can be driven by distinct evolutionary mechanisms. Some organisms may have evolved regenerative capabilities as a response to environmental pressures, such as predation or habitat disturbance, while others may have lost these abilities due to changes in their ecological niche or developmental constraints.
3. **Genetic and Developmental Factors:** The genetic and developmental mechanisms underlying regeneration can vary among different species. Studies have identified conserved signaling pathways and genetic networks involved in regeneration across diverse organisms, but the specific genes and regulatory elements controlling this process can differ significantly. Understanding the molecular basis of regeneration in different taxa can shed light on the factors contributing to its variation.
4. **Ecological and Environmental Influences:** Environmental factors, such as temperature, nutrient availability, and exposure to toxins or pathogens, can influence an organism's regenerative capacity. Additionally, ecological interactions, such as competition and predation, may exert selective pressures that shape the evolution of regeneration in specific environments.
5. **Developmental Plasticity and Life History:** The ability to regenerate tissues or organs may be linked to an organism's life history traits and developmental plasticity. Species with complex life cycles or high rates of tissue turnover may have evolved more robust regenerative abilities to support growth, development, and survival.

By addressing these points and integrating findings from evolutionary biology, developmental genetics, and ecological research, scientists can gain a comprehensive understanding of the factors driving the variation in regeneration capacity among

metazoans. This knowledge is essential for elucidating the evolutionary trajectories of regenerative processes and may have implications for fields such as regenerative medicine and conservation biology.

The ability to regenerate the whole body is present in all basal metazoan lineages and in several lophotrochozoan and deuterostome phyla. Probably the ancestors of those phyla were all capable of extensive regeneration (Fig.1). Regeneration may first arise in early animals, possibly coincident with the origin of multicellularity. This is supported by widely shared features in the process even across divergent species and structures (Bely and Nyberg, 2010).

By what evolutionary mechanism did regeneration first originate? The epiphenomenon hypothesis argue that regeneration was originally a natural consequence of an accessible developmental program that could be automatically redeployed when a structure was lost. In diverse animals, important parallels are evident between regeneration and asexual reproduction (Martinez, Menger and Zoran, 2005; Kawamura *et al.*, 2008; Burton and Finnerty, 2009), tissue homeostasis and growth (Bely and Wray, 2001; Gurley, Rink and Alvarado, 2008), and even embryogenesis (Ghosh *et al.*, 2008; Nakamura *et al.*, 2008).

However, recent studies about changes of chromatin dynamics during regeneration in zebrafish heart and *Drosophila* wing imaginal disc regeneration shows that there are chromatin regions with higher accessibility (putative enhancers) in regeneration. Most of these enhancers are accessible also during normal embryonic development, but some of them became accessible only after damage is inflicted to the tissue (Goldman *et al.*, 2017; Vizcaya-Molina *et al.*, 2018). These observations might challenge the epiphenomenon hypothesis, as they present a molecular event that occurs specifically in regeneration but not in development.

From an evolutionary point of view, regeneration would be maintained if, first, the structure is frequently lost in the field (Griffen *et al.*, 2020). Second, absence of the structure should result in significant fitness-related costs to the individual (Goss, 1969) and third, if the benefits of replacing the lost structure outweigh the costs of regeneration (reviewed in Bely and Nyberg, 2010).

On the other hand, loss of the regeneration ability has been common across animal phylogeny. This could have several evolutionary explanations: The tissue is uncommonly lost in the field (Baumiller and Gahn, 2004; Bateman and Fleming, 2009), regeneration of trivial structures will have no benefit, whereas the loss of crucial structures leads to death (Brautigam and Persons, 2003; Guffey, 2011). If previous tight pleiotropic interactions between regeneration and another developmental process break down and it is no longer under selection pressure (neutral loss) (Galis, Wagner and Jockusch, 2003).

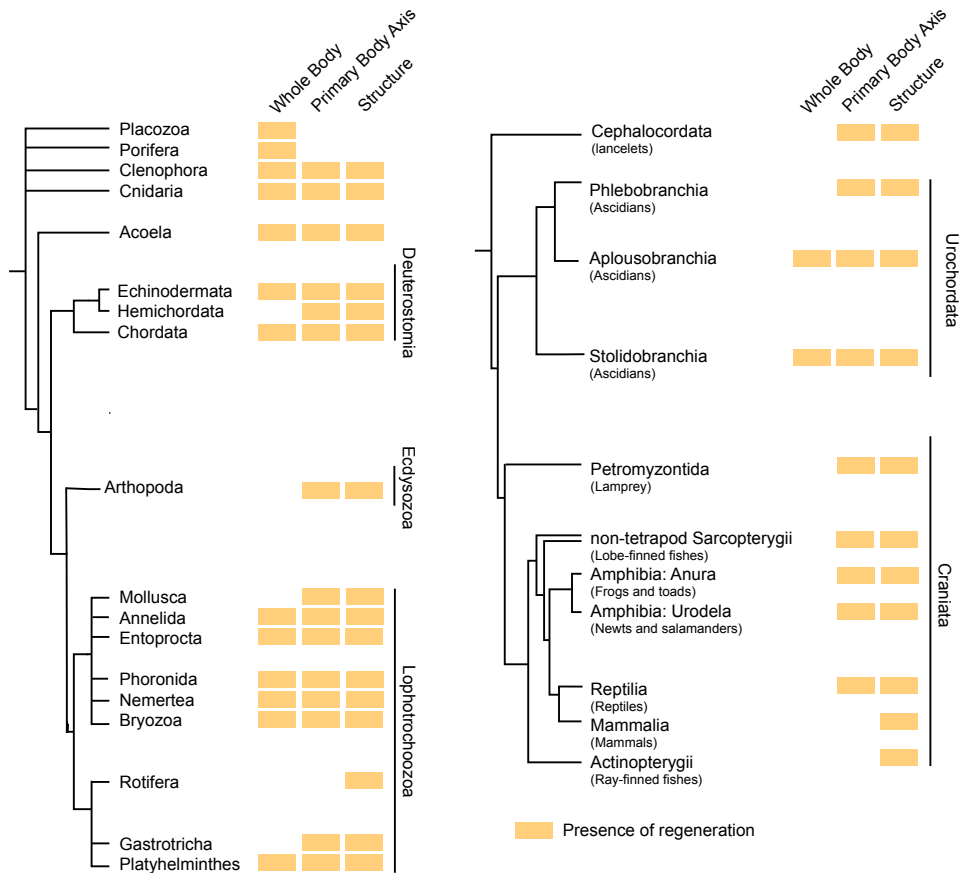


Fig.1. Phylogenetic distribution of regeneration across the Metazoa and the Chordata. ‘Presence of regeneration’ indicates that one well-documented evidence exists for regeneration in that taxon and does not imply that all species in that taxon regenerate. ‘Whole-body regeneration’ is the ability to reconstruct every part of the body (although not necessarily simultaneously or from a tiny fragment). The ability to regenerate the primary body axis is scored independently for each taxon and does not assume homology of body axes across or within phyla. (Data and tree topologies adapted from Bely and Nyberg, 2010).

1.7 Regeneration in the clade Ecdysozoa

Historically, the clade Ecdysozoa has been an example of loss or reduced regeneration capability (Minelli, 2003). Ecdysozoa comprises segmented arthropods (chelicerates, myriapods, crustaceans, and hexapods). It is the most diverse animal lineage that inhabits all aquatic habitats and has conquered land and air during its impressive evolutionary radiation (Giribet and Edgecombe, 2019). It is argued that this loss is due to the appearance of a protective exoskeleton that has had an antagonistic effect on the regenerative capabilities of ecdysozoans (Bely, 2010).

The ability to restore the primary body axis (anterior and posterior body regions and body segments) is lack in this clade (Minelli, 2003; Bely and Nyberg, 2010). But, recently, opposing this long-standing paradigm, it has been shown that the sea spider *Pycnogonum litorale* reestablish the posterior body pole after transverse amputation and can regrow complete segments and the terminal body region (Brenneis *et al.*, 2023). This may potentially represent an ancestral trait, given that the pycnogonid lineage diverged near the base of the arthropod tree (Giribet and Edgecombe, 2019; Ballesteros *et al.*, 2021, 2022). Furthermore, this ability would not only be exceptional for Arthropoda (Bely, 2010; Maruzzo and Bortolin, 2013; Minelli, 2020).

It is also well documented that various arthropod lineages can regenerate appendages (Przibram and Przibram, 1907; Nakamura *et al.*, 2008; Alwes, Enjolras and Averof, 2016; Minelli, 2020) and, in some taxa, even follow self-amputation of limbs (autotomy) during molting complications or to escape predation (Maciej Serda *et al.*, 2022). That is why many arthropods have served historically as model organisms for regeneration research: *Tribolium castaneum*, *Gryllus bimaculatus*, *Blattella germanica* and *Periplaneta americana*, as well as crustaceans such as *Uca pugilator* and the *Parhyale hawaiiensis* (Das, 2015; Khan, Schuster and Smith-Bolton, 2016).

However, if there is one species of arthropod that has been widely used in the study of regeneration, it is the fruit fly, *Drosophila melanogaster*, particularly the regeneration of larval organs and adult gut (Worley, Setiawan and Hariharan, 2012; Zhang and Edgar, 2022).

2. *Drosophila melanogaster* as an animal model to study regeneration

Drosophila belongs to the Insecta class and the Diptera order. *Drosophila*, as other holometabolous insects, cannot regenerate appendages after metamorphosis, because regenerative capacity of the appendages or appendage primordia of these animals is constrained by the ability of each to grow (Hariharan, Wake and Wake, 2015).

Much of regeneration studies in *Drosophila* has focused on the imaginal discs, which are the larval primordia of adult structures (Hariharan and Serras, 2017; Ahmed-De-Prado and Baonza, 2018). However, recent studies put the focus on stem cell-containing tissues in the adult, such as the midgut (Jiang *et al.*, 2011; Patel *et al.*, 2019; Tamamouna *et al.*, 2021), germline (Brawley and Matunis, 2004; Kai and Spradling, 2004), muscle (Chaturvedi *et al.*, 2017; Boukhatmi and Bray, 2018) and the brain (Fernández-Hernández, Rhiner and Moreno, 2013; Moreno *et al.*, 2015). *Drosophila* is also useful to study responses that occur after injury, such as wound healing (Razzell, Wood and Martin, 2011; Zulueta-Coarasa and Fernandez-Gonzalez, 2017; Tsai, Wang and Galko, 2018) and transdetermination (Worley, Setiawan and Hariharan, 2012; Beira and Paro, 2016).

Drosophila has many advantages as a model organism to study regeneration. It is easy and cheap to maintain, it has a rapid life cycle (~ 10 days from egg to adult at 25°C) and a short lifespan of approximately 40 days at 25°C which allows to monitor regenerative growth soon after damage. In addition, the diversity of genetic tools which are available in *Drosophila* allows to manipulate targeted gene expression and efficient generation of genetic mosaics, facilitating in-depth mechanistic studies of regeneration. Additionally, *Drosophila* offers the possibility to conduct genetic screens that can be used to identify genes that drive regeneration (Belacortu and Paricio, 2011; Hariharan and Serras, 2017; Fox, Cohen and Smith-Bolton, 2020).

2.1 Imaginal discs development

Imaginal discs precursors are small groups of ectodermal cells which proliferate extensively and invaginate to form the imaginal discs (including the wing discs) during larval development. By the end of larval stages these cells are committed to specific fates. During metamorphosis, while larval tissues are disappearing by apoptosis, the imaginal discs undergo major morphogenetic changes and finally evert to form the adult structures (Pastor-Pareja *et al.*, 2004). There are in total 19 discs in the larva. Wing appendage in the adult fly comes from the 8th pair of imaginal discs (Held LJ, 2005).

Wing imaginal discs are epithelial sacs of cells present in larval stages that gives rise to the adult wing, wing hinge, and also the dorsal half of the body wall in the second thoracic segment of the adult fly (Tripathi and Irvine, 2022). At the cellular level, the wing disc is formed by: (1) An outer layer, the peripodial membrane, with squamous cells that provide little contribution to the cuticular structures in the adult (Haynie and Bryant, 1986). (2) The disc *per se* is formed by a single columnar pseudostratified epithelial monolayer, which confers advantages for imaging and analysis of structural changes in tissue and cell shape upon manipulations (Held LJ, 2005) (Fig.2).

In addition to the epithelia cells, wing discs also contain a small number Neurons and associated glia embedded within the epithelium (Huang, Dambly-Chaudière and Ghysen, 1991; Gómez-Skarmeta, Campuzano and Modolell, 2003), adult muscle precursor cells (Mandaravally Madhavan and Schneiderman, 1977; Everetts *et al.*, 2021) and tightly associated tracheal cells (Inoue and Hayashi, 2007).

The wing disc have three lineage restrictions (Garcia-Bellido, Ripoll and Morata, 1973): (1) The subdivision into Anterior (A) and Posterior (P) compartments which is established during embryogenesis (Lawrence and Morata, 1977; Martinez-Arias and Lawrence, 1985; Dahmann and Basler, 1999), molecular mechanisms underlying A and P identities were found to require the key gene *engrailed (en)* (Garcia-Bellido and Santamaria, 1972; Morata and Lawrence, 1975; Lawrence and Struhl, 1996).

(2) The Dorsal (D) and Ventral (V) compartments. Gene *apterous (ap)* is crucial for dorsal cells identity (Diaz-Benjumea and Cohen, 1993).

(3) The trunk region (Notum, N) and the appendage (Wing pouch plus Hinge, WH) regions (Garcia-Bellido, Ripoll and Morata, 1973; Morata and Lawrence, 1979; Diaz-Benjumea and Cohen, 1993). The N will form the thoracic body wall of the adult and the WH the wing and the hinge. Vein/EGFR signaling makes N cells distinct from and immiscible with WH cells (Zecca and Struhl, 2002). It is worthy to outline here that genetic programs in charge of conferring compartment identity are stable through development.

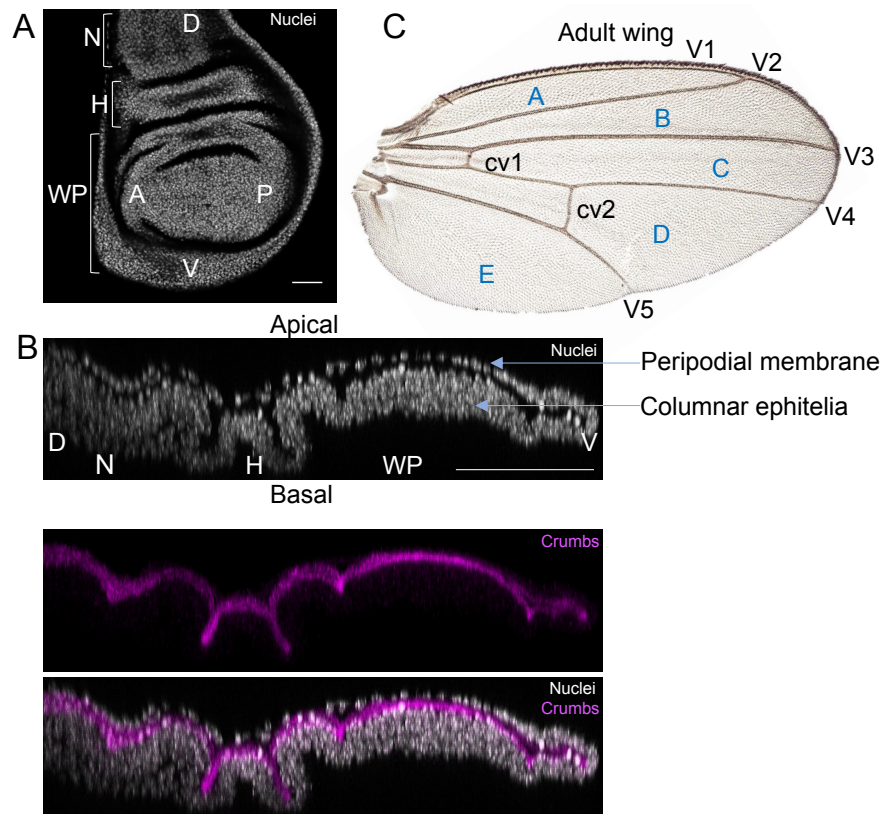


Fig.2. The wing imaginal disc and the adult wing of *Drosophila melanogaster*. (A) Nuclear staining of a wing imaginal disc with different regions depicted (N=Notum, H=Hinge, WP=Wing Pouch, A=Anterior, P=Posterior, D=Dorsal, V=Ventral). (B) Orthogonal view of the disc showing two different layers of the polarized epithelia: Peripodial membrane, at the apical side, and the columnar pseudostratified-epithelia, basal. Immunostaining for the apical polarity gene *crumbs* that co-localizes with the apical side of the epithelia (merged). Nuclei staining: TP3, scale bars=50µm. (C) An adult female wing from the Canton S strain, with veins (V), cross-veins (cv) and interveins (A-E) depicted.

2.2 The wing imaginal disc, a model system to study regeneration

2.2.1 When to regenerate

During the third larval stage, cell fates in the imaginal disc epithelia are specified. But, until the entering into metamorphosis cells do not fully differentiate (Held LJ, 2005). Proliferation in wing imaginal disc stops shortly after pupariation (Graves and Schubiger, 1982; Schubiger and Palka, 1987; Milán, Campuzano and García-Bellido, 1996; Buttitta *et al.*, 2007). The temporal window for imaginal discs to regenerate is through most of the third larval instar stage, but this ability ceases with metamorphosis.

2.2.2 Early studies on imaginal disc regeneration: Fragmentation and culture.

In the 60's and 70's by Hardon and Bryant showed that after cutting a piece of the disc, a regeneration blastema was formed (Hadorn *et al.*, 1968), and when isolated this can regenerate the lost portion (Karpen and Schubiger, 1981). Also, it has been demonstrated that after irradiation (resulting in 50% of imaginal discs cells death) normal flies still arising (Haynie and Bryant, 1977).

The classical method for studying regeneration was microsurgical fragmentation of discs. Fragmented discs were implanted into an anesthetized host fly abdomen using a micropipette. Those flies were culture in vials for several days and later on recovered to analyse regeneration (Hadorn *et al.*, 1968; Oberlander, 1972; Ursprung, 2004). These fragments were also transplanted into larvae to score the differentiation potential after metamorphosis (Chan and Gehring, 1971).

These classic studies demonstrated: (1) Disc fragments regenerated the missing part by localized cell proliferation (Hadorn and Buck, 1962) and the isolated blastemas were shown capable of regenerating the lost structures (Karpen and Schubiger, 1981). (2) Confrontation of blastemas from distant parts induced intercalary growth (Haynie and Bryant, 1976). (3) Certain types of disc fragments generated mirror-image duplications instead of the missing portion (Bryant, 1971; Schubiger, 1971), likely because the lack of some positional cues. (4) Blastemas tend to change fate during regeneration (transdetermination) (Hadorn, 1965; Schubiger and Hadorn, 1968; Hardon, 1978).

2.2.3 Genetic ablation, a new era

There are many technical difficulties associated to this fragmentation experiments that impeded the progress of the field for many years. But recently, the development of new genetic tools to ablate tissues in a spatially and temporary control manner have fuelled again the interest in imaginal discs regeneration (Smith-Bolton *et al.*, 2009; Bergantiños, Corominas and Serras, 2010)

Genetic ablation of wing imaginal discs offers advantages over the traditional approach. Since the discs are ablated *in situ*, they are able to generate the adult wing and thus the extent of regeneration can be assessed in this adult appendage. Also, the combination of genetic ablation with other genetic tools (ex. knockdown experiments) facilitates the discovery of novel regulators of regeneration (Hariharan and Serras, 2017).

Binary expression systems that allow spatial control of cell ablation have been widely used. The Gal4/UAS system driven by tissue-specific promoters (Fig. 3) (Brand and Perrimon, 1993), is used to express pro-apoptotic genes, such as *eiger* (*egr*), *reaper* (*rpr*) or *head involution defective* (*hid*), to ablate restricted portions of the wing disc (Smith-Bolton *et al.*, 2009; Bergantiños, Corominas and Serras, 2010; Repiso, Bergantiños and Serras, 2013; Martín, Pinal and Morata, 2017)

Regulators of the binary system add temporal control to the ablation process. The temperature-sensitive (ts) repressor Gal80^{ts} (Fig. 3) (McGuire, Mao and Davis, 2004) has been used to restrict ablation to the appropriate time window during larval development (Bergantiños, Corominas and Serras, 2010)

But the Gal4/UAS system has some disadvantages. For example, the impossibility to injure a tissue and to query the function by knocking down or misexpression of a candidate regeneration gene at the same time in a population of cells different from the ones that are being ablated (Fox, Cohen and Smith-Bolton, 2020).

There are several genetically engineered tools that allow independent expression of transgenes in the wing disc. The modified binary system LexA-hinge-Gal4 activation domain (LHG) /LexO with a Gal4 hybrid LHG transcription factor (TF) regulable by Gal80^{ts}

(Fig. 3) achieves spatial separation of the expression of a pro-apoptotic gene for cell ablation and, via a Gal4/UAS induced transgene, genetic manipulation of a regeneration factor (Santabárbara-Ruiz *et al.*, 2015)

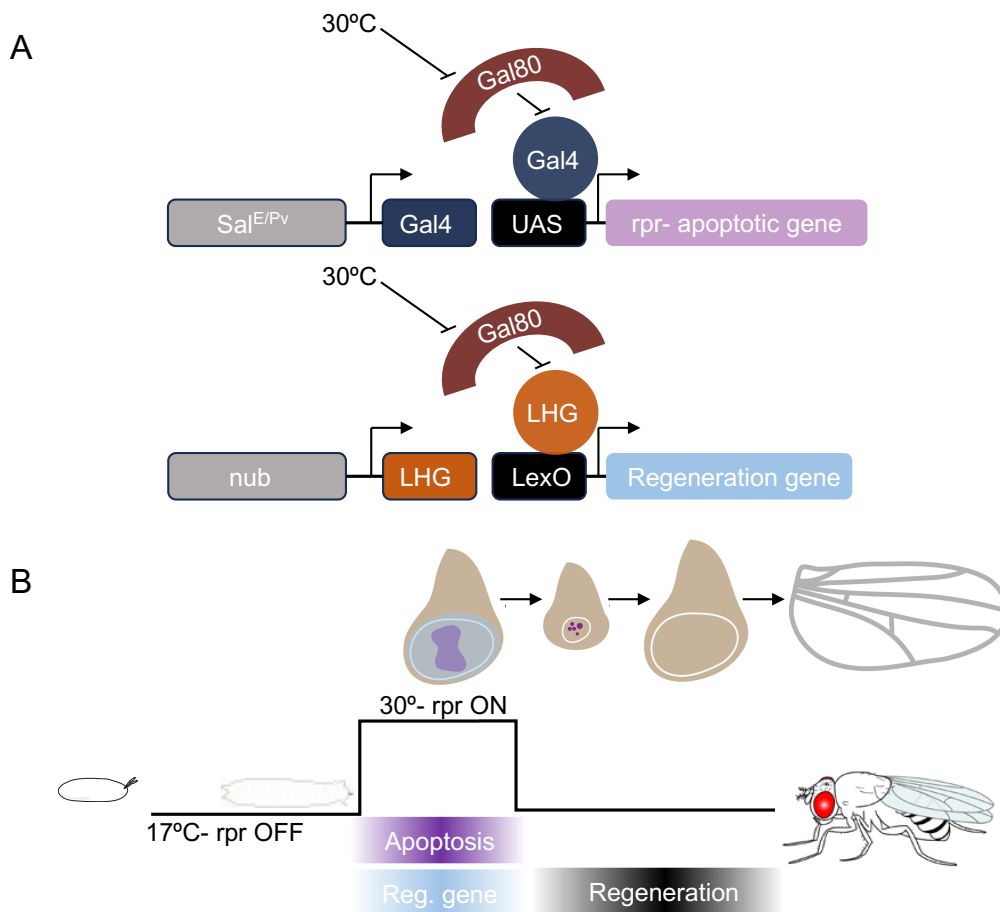


Fig 3. The dual inducible trans-activator system Gal4>UAS/ LHG>LexO. (A) At 17°C Gal80 is active and blocks Gal4 and LHG activity. However, at 30°C Gal80 is inactive and Gal4 and LHG can bind to UAS/ LexO sequences, respectively. This promotes the expression of the pro-apoptotic gene *rpr* (purple) and an *RNAi* or gain of function of a gene (blue) of interest for regeneration during the same temporal window. (B) Scheme that shows the time course of cell death and regeneration. Cell death starts the 8th day (192h after egg laying) when larvae are transferred from 17°C to 29°C for 11 hours. Then, they are shifted back to 17°C to allow tissue recovery. When adult flies emerge, wings are analyzed to detect defects in pattern and size.

The Q binary system in combination with Gal4/UAS permits to control ablation and transgene expression separately (Bellen *et al.*, 1992) . For example, using the Q system to drive the expression of a receptor for a toxin that is fed to the animal to accomplish temperature-independent injury, meanwhile Gal4 drives transgene expression (Obata *et al.*, 2015).

Another tool is the dual expression method for induced site-specific eradication (DEMISE), which requires just one binary expression system. This approach allows raising animals at 25°C (shortening the experiments) (Cohen *et al.*, 2018).

Recently, optogenetic methods are being used to perform temporal control of tissue ablation (Makhijani *et al.*, 2017). This emerging tool will enable large-scale screens in the background of a precision injury setting (di Pietro *et al.*, 2021). Moreover, the field has now large toolkit collections of *Drosophila* RNAi (Dietzl *et al.*, 2007; Perkins *et al.*, 2015) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) (Li-Kroeger *et al.*, 2018; Meltzer *et al.*, 2019) lines, that can be used to elucidate regeneration mechanisms further.

2.3 How to regenerate an imaginal disc?

2.3.1 Programmed cell death: Apoptosis in imaginal discs

Programmed cell death (apoptosis) is a process by which cells triggers their own elimination. It involves the activation of several cysteine-proteases, known as caspases, which kill the cells by destroying protein substrates. Apoptosis is conserved in all metazoans, although the initiation events vary among species (Fuchs and Steller, 2011; Denton, Aung-Htut and Kumar, 2013; Savitskaya and Onishchenko, 2015). Apoptosis might be developmentally programmed, such as in the tail structures in amphibians, the inter-digital tissues in vertebrates or the joints of *Drosophila* legs (Lindsten *et al.*, 2000; Igaki, 2009; Ishizuya-Oka, Hasebe and Shi, 2010). But in other contexts, there is non-programmed apoptosis, such in response to stress mechanisms, damage and elimination of aberrant cells (Norbury and Hickson, 2001). In *Drosophila*, apoptosis is initiated by the activation of one or more pro-apoptotic genes (*hid*, *rpr*, *grim*) that block the activity of the *Drosophila* inhibitor of apoptosis1 protein (Diap1). The loss of Diap1 function allows the activation of the caspases and initiation cell death (Fuchs and Steller, 2011; Pinal, Calleja and Morata, 2019).

2.3.2 Wound healing

Imaginal discs heal very efficiently. Transient heterotypic contacts between edges of the injury appear during the first day of *in vivo* culture in two cell layers of the disc.

Closing the wound requires cytoskeleton rearrangements: Contact between the edges by filopodia and closure facilitated by an actin-rich cable (Reinhardt, Hodgkin and Bryant, 1977; Reinhardt and Bryant, 1981; Bryant and Fraser, 1988; Bosch *et al.*, 2005; Zulueta-Coarasa and Fernandez-Gonzalez, 2017) It has been also demonstrated the importance of Plexin A in regulating early stages of wound closure (Yoo *et al.*, 2016). Within 48 hours in culture, the wound is perfectly closed, and the tissue shows a restored continuity.

2.3.3 Early signals

After damage there is a release of early signals coming from dying cells. Ca^{2+} waves have been shown to appear upon injury in *Drosophila* embryos (Narciso *et al.*, 2015). After Imaginal disc injury, intracellular Ca^{2+} propagates via gap junctions and have been described to activate the Nicotinamide Adenine Dinucleotide Phosphate (NADPH) dual oxidase (DUOX) which acts as a source of H_2O_2 (Narciso *et al.*, 2015; Restrepo and Basler, 2016). Reactive Oxygen Species (ROS) produced rapidly after damage are necessary for regeneration and crucial for the activation of c-Jun N-terminal kinases (JNK) and p38

stress-activated protein kinases (explained extensively below) (Narciso *et al.*, 2015; Fogarty *et al.*, 2016).

2.3.4 Signaling pathways

Early signals turn on different signaling pathways that orchestrates the next steps of regeneration: Upregulation of the Wnt protein Wingless (Wg) (Gibson and Schubiger, 1999; McClure, Sustar and Schubiger, 2008; Smith-Bolton *et al.*, 2009; Katsuyama *et al.*, 2015) is crucial for regeneration. After damage JNK triggers the activation of a wing-specific enhancer in the *wg* gene which contain five predicted AP1 binding sites (Gracia-Latorre *et al.*, 2022). Wg increases Myc levels via a double-repression mechanism involving Notch during regeneration (Smith-Bolton *et al.*, 2009).

Hippo pathway contributes to regeneration in imaginal discs (Grusche *et al.*, 2011; Repiso, Bergantiños and Serras, 2013). Following tissue damage, The LIM-domain protein Ajuba (Jub) (likely in response to changes in cell tension) inhibit the Hippo pathway, resulting in increased Yorkie (Yki) activity (Sun and Irvine, 2011; Meserve and Duronio, 2015).

The JAK/STAT pathway is also important for regenerative growth (Pastor-Pareja, Ming and Tian, 2008; Katsuyama *et al.*, 2015; La Fortezza *et al.*, 2016; Verghese and Su, 2016; Santabárbara-Ruiz *et al.*, 2019). The activation of the JAK/STAT pathway requires previous expression of Unpaired (Upd) cytokines (the ligand upstream of the pathway). JNK (Pastor-Pareja, Ming and Tian, 2008; Katsuyama *et al.*, 2015; Santabárbara-Ruiz *et al.*, 2015) and the p38 (Santabárbara-Ruiz *et al.*, 2015) kinases are key for Upd expression.

As far it has been demonstrated that Yki can activate *Myc* expression, crosstalk between these pathways is likely to occur during regeneration (Neto-Silva, de Beco and Johnston, 2010).

2.3.5 Proliferation

After surgical damage and *in vivo* culture, a proliferation blastema around the wound appears. (Dale and Bownes, 1980; Abbott, Karpen and Schubiger, 1981; O'Brochta and Bryant, 1987). After genetic ablation of sufficient mass of cells in the disc a localized mass of dividing cells has been detected, which resembles the blastemas found after physical injury (Worley *et al.*, 2022). this observation has been confirmed. A blastema forms after apoptosis occurs and lost tissue is replaced by localized proliferation of the adjacent cells (Fig. 4), without any evidence for a population of progenitor cells (Bosch, Baguñà and Serras, 2008; Smith-Bolton *et al.*, 2009; Bergantiños, Corominas and Serras, 2010; Worley *et al.*, 2022).

The rate of cell proliferation increases during regenerative growth compared to normal growth (Wells, Yoshida and Johnston, 2006; Gerhold *et al.*, 2011). Proliferating cells re-orient their axes of divisions (compared to the proximodistal orientation in the wing pouch during normal growth) to facilitate the efficient replacement of lost tissue (Repiso, Bergantiños and Serras, 2013). Cell proliferation starts before the complete closure of the wound reinforcing the vision of direct stimulation of proliferation by the damaged tissue itself. (Karpen and Schubiger, 1981; Kiehle and Schubiger, 1985; Bryant and Fraser, 1988; Mattila *et al.*, 2005; Bosch, Baguñà and Serras, 2008)

While the imaginal disc is proliferating, a suppression mechanism of proliferation elsewhere occurs: Nitric Oxide Synthase (Nos) activity in the prothoracic gland drops the systemic levels of ecdysone needed for disc growth (Jaszczak et al., 2015). A key finding was the discovery of the Insulin-like peptide 8 (Ilp8), a member of the insulin/relaxin superfamily, which is secreted by imaginal discs to the haemolymph in response to damage to delay pupariation (Colombani, Andersen and Léopol, 2012; Garelli et al., 2012).

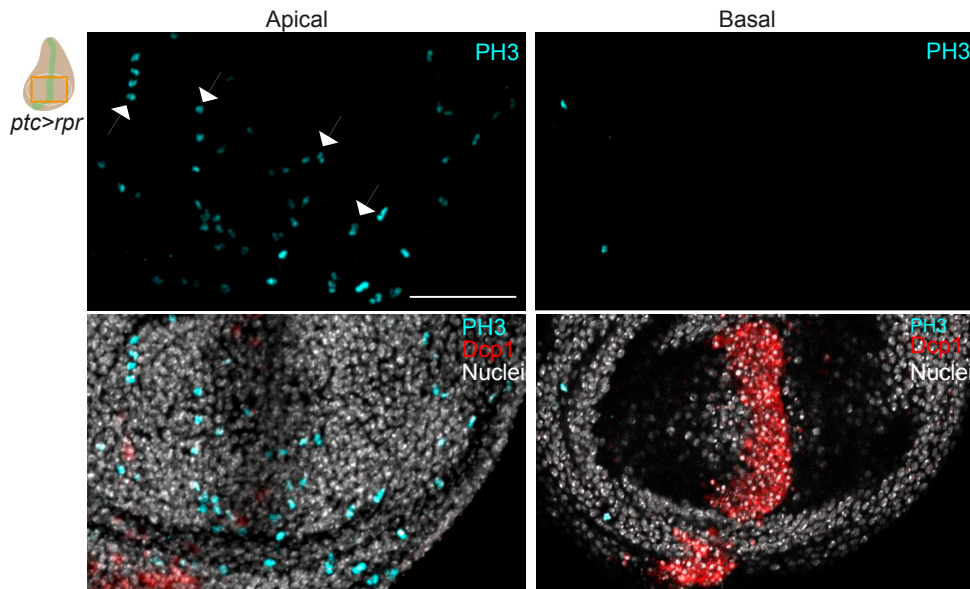


Fig. 4. Localized proliferation in the wing imaginal disc after genetic ablation. Details of the WP of an imaginal disc. Right panel, Apical: Mitotic cells detected by anti-phospho histone 3 antibody (PH3) localize in the apical side of the imaginal disc in rows (white arrows) surrounding the ablated domain (*ptc* region, depicted in green in the cartoon). Left panel, Basal: In the basal region of the disc the dying cells are being extruded (*Drosophila* cleaved caspase 1 (Dcp1) staining marks apoptotic cells, red) and few dividing cells are detected. Nuclei was stained using TP3.

2.3.6 Re-patterning

Growth and expression of pattern morphogens (such as Wg and Decapentaplegic (Dpp)) have been well characterized during normal development. Upon ablation, the pattern of expression of these morphogens is disrupted and not recovered until regenerative growth is complete (Smith-Bolton et al., 2009).

Also, upon damage, there is loss of cell fate specification, as observed by the lack of expression of patterning and cell fate genes such as the transcriptional activator *vestigial* (*vg*) and markers for adult wing intervein and vein identity (Smith-Bolton et al., 2009; Díaz-García and Baonza, 2013).

Re-specified cells and their progeny adopt new identities to replace lost cell types (Repiso, Bergantiños and Serras, 2013) even crossing A-P compartment boundary under extreme conditions (Herrera and Morata, 2014). In addition, it has been described that cells from the hinge of the wing disc appear capable of generating cells that contribute to the wing pouch (Smith-Bolton et al., 2009; Herrera, Martín and Morata, 2013; Verghese and Su, 2016). Damage dependent JNK activation contributes to changes in cell fates by reducing the activity of Polycomb-dependent silencing (Lee et al., 2005). Also,

the chromatin regulator Taranis has been shown to stabilize compartmental identities during regeneration (Schuster and Smith-Bolton, 2015).

3. Early signals in regeneration: ROS

ROS are derivatives of oxygen that appears as a by-product of normal aerobic metabolism. Classically ROS have been considered as toxic cellular by-products. Indeed, high levels of ROS in cells can cause irreversible oxidation events in biomolecules, such as DNA, lipids, and proteins, and eventually causing cell death, damage and various diseases and the development of diseases, as reviewed in (Lennicke and Cochemé, 2021a) . However, currently there are plenty of evidences that low levels of ROS are beneficial for the cells and tissues (Schieber and Chandel, 2014; Milkovic *et al.*, 2019).

All cells have different antioxidant systems to maintain ROS at low levels. Reduced NADPH, is a key redox cofactor for antioxidant enzymes, such as Glutathione Reductase (GR), and also for ROS-generating NADPH oxidases (NOXs). Therefore, cellular redox state and cellular metabolism are firmly interconnected (Sies and Jones, 2020).

The most physiologically relevant ROS are the superoxide anion radical ($O_2^{\bullet-}$) and non-radical H_2O_2 , these molecules are generated sequentially.

3.1 The $O_2^{\bullet-}$ and H_2O_2 species

$O_2^{\bullet-}$ represents the superoxide anion, which is negatively charged oxygen molecule with an extra electron. It is produced by specific enzymes, including NOXs (Lambeth, 2004) , or by complexes I and III (CI and CIII) of the mitochondrial Electron Transport Chain (ETC) by single-electron reduction of O_2 (Murphy, 2009). Levels of $O_2^{\bullet-}$ in cells are usually low because of its spontaneous and/or enzyme-mediated transformation to H_2O_2 catalysed by superoxide dismutases (Sod1-3) (Zelko, Mariani and Folz, 2002)

H_2O_2 represents hydrogen peroxide, a chemical compound composed of two hydrogen atoms and two oxygen atoms. It is a clear liquid at room temperature and has strong oxidizing properties. It presents high stability compared with other ROS. H_2O_2 exhibits low overall reactivity but, importantly, high selectivity for the thiol group of cysteine residues (Veal, Day and Morgan, 2007). H_2O_2 moves across membranes by simple diffusion or transported via channels, such as aquaporins (Bienert and Chaumont, 2014). Xanthine oxidases, NOX4 and monoamine oxidases are in charge of direct H_2O_2 production (Kelley *et al.*, 2010; Nisimoto *et al.*, 2014). H_2O_2 removal is mediated by heme-dependent Catalase (Cat) (Nandi *et al.*, 2019), selenium-dependent Glutathione peroxidase (GPx) (Brigelius-Flohé and Maiorino, 2013), and thiol-containing Peroxiredoxin (Prx) (Day *et al.*, 2012).

3.2 Biological functions of ROS

In the last few years, more and more evidences demonstrate that ROS have key roles in regulating many aspects of cell function. ROS are in charge of post-translational modifications of proteins, changing its localization and function in a regulated and reversible manner (Paulsen and Carroll, 2013; Holmström and Finkel, 2014). All these

events are called redox signaling and controls many aspects of cell function, from tuning the activity of metabolic enzymes and TFs to regulating gene expression and epigenetic modifications. Moreover, ROS control processes at the organ and organismal level, such as wound healing, extracellular matrix formation, and immunity (Sen and Roy, 2008; Roberts, 2017; Singla, Holmdahl and Csanyi, 2019)

3.3 Cysteine redox biochemistry

Cysteines (Cys) are the least abundant amino acid in proteins, but a high proportion of this residue (~90%) are evolutionarily conserved. This has been associated to their involvement in redox signaling, as many ROS mediate their biological functions through the reversible oxidation of thiol groups on Cys. (Fomenko, Marino and Gladyshev, 2008; Marino and Gladyshev, 2010; Cremers and Jakob, 2013). Cys oxidation acts as a “redox switch,” which can alter the function, localization, activity, and/or interaction with binding partners of the target protein.

These residues exist in a reductor environment as protonated thiols (R-SH) and become deprotonated thiolate (R-S⁻) form upon oxidation. Thiolates are highly reactive and undergo redox reactions, resulting in a wide range of oxidative post-translational modifications (Matsui *et al.*, 2020; Keenan, Zachman and Hirschey, 2021). Several factors contribute to the sensitivity of Cys to oxidation: The low acid dissociation constant (pKa) of redox-sensitive thiolates, accessibility to oxidants, the reduction potential, and the surrounding microenvironment, including neighbouring charged amino acid residues (Poole, 2015; Matsui *et al.*, 2020; Xiao *et al.*, 2020).

Some Cys are structural and used to be buried inside the protein tertiary structure forming intramolecular disulphides. This class of Cys could become exposed and undergo oxidation upon protein conformational changes (Behring *et al.*, 2020).

On the other hand, exposed Cys (located at the protein surface) are more prone to be redox-active because their accessibility to external signals and are key for the formation of mixed disulphides with binding partners. Two Cys residues in close proximity in the tertiary structure indicates oxidation susceptibility (Sanchez *et al.*, 2008).

3.4 ROS, an early signal during wing imaginal disc regeneration in *Drosophila*

In *Drosophila* imaginal disc regeneration ROS is quickly produced by dying cells upon genetic ablation by the activation of pro-apoptotic genes such as *rpr* (Fig. 4) (Santab rbara-Ruiz *et al.*, 2015). This has been demonstrated through the use of ROS indicators (i.e., CellROX) that are sensitive to ROS of general origin. Rpr is present in mitochondrial membranes and promotes mitochondrial fragmentation by binding to the pro-fusion Marf protein. (Varkey *et al.*, 1999; Thomenius *et al.*, 2011; Clavier *et al.*, 2016). ROS of mitochondrial origin is accumulated upon *rpr* induction (detected with MitoSox probe). An amplification loop in apoptotic cells consisting in JNK further activation of the gene *rpr* (Shlevkov and Morata, 2012) also contribute to the burst of mitochondrial ROS coming from dying cells.

Low levels of ROS detected in the living cells near the damaged area are a key early signal in the onset of wing imaginal disc regeneration (Fig. 4), as far as scavenging of ROS using

chemicals (i.e., N-Acetyl-L-Cysteine (NAC)) or enzymatic antioxidants (overexpression of Sod1 or Cat transgenes) blocks the regeneration process (Santabábara-Ruiz *et al.*, 2015).

3.5 Propagation of ROS to regenerating cells

ROS appears as a burst in the apoptotic cells but it was found also in low levels in living cells surrounding the damaged area (Fig 4). There two explanations for this observation: (1) ROS can be propagated to the living cells near the dying zone by aquaporins (Bienert *et al.*, 2007) or gap junctions (Feine *et al.*, 2012) present in the imaginal disc epithelial cells. Also, it has been proposed that simple diffusion through the intercellular space could contribute to ROS spreading. (2) Calcium waves are induced upon wounding and spread through gap junctions activating DUOX in the living cells (Razzell *et al.*, 2013) This will result in H₂O₂ production by oxidases that are not mitochondrial.

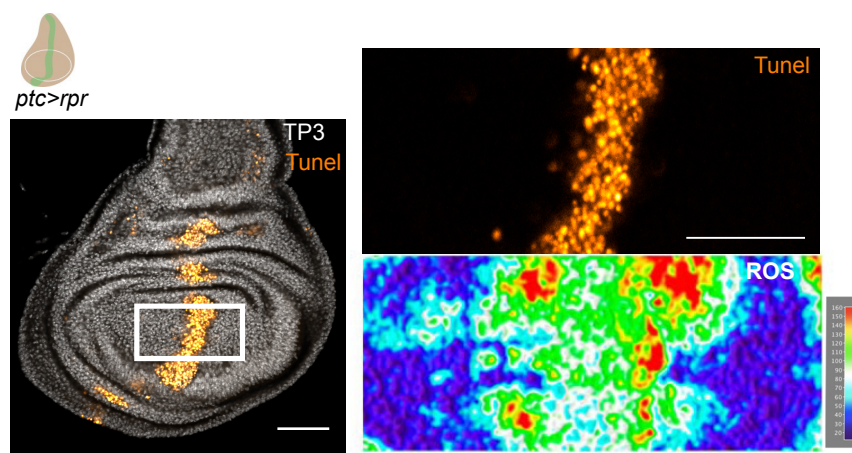


Fig. 5. ROS detection after genetic ablation of an imaginal disc. An imaginal disc in which apoptosis has been induced in the *ptc* region (green in the cartoon) (left pannel). Right panel: Details of the WP. Cell death detected by Tunel staining (up) and ROS (down) with the Mitosox Dye (Thermal LUT, scale indicated). Note that high levels of ROS are detected in the central region, co-localizing with cell death, while low levels spread to the nearby living cells. TP3 were used to stain nuclei. Scale bars=50µm. Adapted from (Santabábara-Ruiz *et al.*, 2015).

4. Mitogen activated protein kinases (MAPKs) activated by stress (SAPKs)

Eukaryotic cells are equipped with diverse intracellular signal transduction systems, which sense various stressors from the external and internal environments (such as ROS) and triggers different cellular responses, including apoptosis, cell cycle arrest, DNA repair and cytokine production (reviewed in Mordente, Ryder and Bekker-Jensen, 2024).

The mitogen-activated protein kinases activated by stress (SAPKs) cascades are key signaling systems, evolutionarily conserved in all eukaryotic cells (Widmann *et al.*, 1999; Kyriakis and Avruch, 2001). Four main SAPK cascades that converge on extracellular signal-regulated kinases 1 and 2 (ERK1/2), ERK5, JNKs, and p38 MAPKs have been well characterized. Each cascade consists of three classes of proteins: MAPK, MAPK kinase (MAP2K), and MAP2K kinase (MAP3K). MAP3K phosphorylates and activates MAP2K, and activated MAP2K in turn phosphorylates and activates MAPK.

ROS stimulates SAPKs such as p38 and JNK.(Santabárbara-Ruiz *et al.*, 2015). A mechanism that would explain this activation could be that the intracellular oxidative environment fosters the activation of kinases upstream of the JNK and p38 pathways. This is the example of the MAP3K Apoptotic signal-regulating kinase 1 (Ask1), which is involved in certain types of receptor-mediated signaling in an ROS-dependent fashion. (Takeda *et al.*, 2008; Son *et al.*, 2013).

5. The MAP3K Ask1

Ask1, also designated as MAP3K5 belongs to the MAP3K family identified in the p38 and JNK pathways (Wang *et al.*, 1996; Ichijo *et al.*, 1997). Ask1 is activated by different stressors including endoplasmic reticulum stress, calcium overload, oxidative stress as well as receptor-mediated inflammatory signals such as Tumor Necrosis Factor- α (TNF α) (Saitoh *et al.*, 1998; Nishitoh *et al.*, 2002; Takeda *et al.*, 2004; Matsuzawa *et al.*, 2005). Upon activation, this protein exhibits serine (Ser)/threonine (Thr) kinase activity.

5.1 Structural features of Ask1

Ask1 possesses a Ser/Thr kinase domain in its midportion flanked by long N- and C-terminal domains that have regulatory functions (Tobiume *et al.*, 1997). The kinase domain (KD) is highly conserved throughout evolution whereas the N- and C-terminal regulatory domains exhibit a fair degree of divergence in the Ask family. It has been shown that activation of Ask1 is regulated by phosphorylation of a threonine (Thr) residue within the KD of Ask1 (Thr806, Thr845 and Thr747 of human, mouse and *Drosophila* Ask1) and that the phospho-specific (phospho-Ask1) antibodies recognizing phosphorylation of this Thr residue monitors the state of activation of this protein (Tobiume, Saitoh and Ichijo, 2002; Santabárbara-Ruiz *et al.*, 2015)

Ask1 has been shown to form a high-molecular-mass (HMM) complex (>1500 kDa) through the C-terminal coiled-coil (CCC) domain in the cell (Noguchi *et al.*, 2005). Many findings suggest that oligomerization-dependent formation of the HMM complex is required for the kinase activity of Ask1 (Tobiume, Saitoh and Ichijo, 2002). As this HMM complex interacts with many regulatory partner proteins and serves as a platform for the regulation of Ask1 activity it has been named in literature as the Ask1 signalosome.

5.2 Regulator partners of Ask1 signalosome

5.2.1 Thioredoxin

Thioredoxin (Trx) is a negative regulator partner of Ask1. Trx is redox regulated through two Cys residues. In a reductor environment Trx is present in the reduced form [Trx-(SH)₂] and binds to the N-terminal segment of Ask1 inhibiting its kinase activity (Saitoh *et al.*, 1998; Liu *et al.*, 2000) by impairing the homophilic interaction through the N-terminal coiled coil (NCC) domain (Fujino *et al.*, 2007). Upon ROS exposure, Trx Cys becomes oxidized [Trx-S₂] and dissociates from Ask1. Then, Ask1 oligomerizes and became active by inducing the phosphorylation of the critical Thr residue in the kinase domain.

5.2.2 TNF receptor associated factors (TRAFs)

Two members of the TRAFs superfamily: TRAF2 and TRAF6 have been identified as positive interactor partners of Ask1. Both are specifically recruited to the Ask1 signalosome in a ROS-dependent manner (Nishitoh *et al.*, 1998; Noguchi *et al.*, 2005). Also, it has been shown that ROS-induced Ask1 activation was strongly suppressed in TRAF2- and TRAF6-deficient mouse embryonic fibroblasts (MEFs) (Noguchi *et al.*, 2005). These findings suggest that, after ROS-induced dissociation of Trx from the inactive Ask1, recruitment of TRAF2 and TRAF6 is required for the homophilic interaction through the NCC domain, a prerequisite for the activation of the Ask1 signalosome.

5.2.3 Postranslational modifications

Posttranslational modifications of Ask1 critically modulate the function this MAP3K. As explained above, phosphorylation of Thr838 residue activates the kinase activity of Ask1. But Ask1 has other negative phosphorylation sites: Ser/Thr kinases of the protein kinase B family (Akt), a Ser/Thr kinase activated by lipid kinase phosphatidylinositide 3-kinase (PI3K) pathway in response to insulin receptor (IR) activation, has been demonstrated to phosphorylate Ser83 of human Ask1 and suppress the kinase activity of Ask1 (also named attenuating phosphorylation by some authors) (Kim *et al.*, 2001; Yuan *et al.*, 2016). This is relevant because ROS have been demonstrated to trigger cell proliferation through PI3K pathway. All the components of the PI3K pathway are highly conserved through evolution and play a crucial role in regulation of growth as well as in sensing nutrient availability. (Nässel and Broeck, 2016).

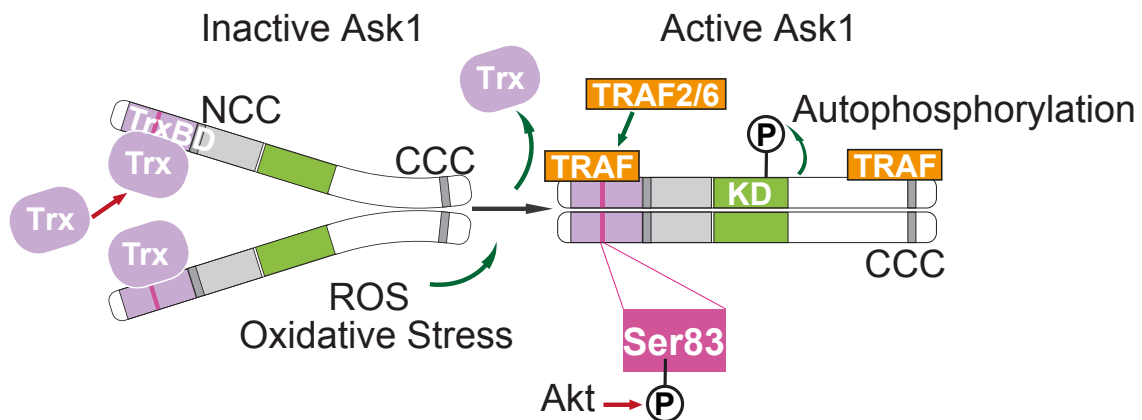


Fig. 6. Ask1 activation is ROS-dependent. Ask1 is activated in a ROS- dependent fashion. In normal conditions, Trx is bound to Ask1, inhibiting its oligomerization through the N- and C- coiled coil domains (NCC and CCC in the cartoon). Upon ROS signaling, Trx is released and Ask1 can oligomerize, recruiting mammalian TRAF2/6 factors and becoming completely active by an autophosphorylation in the kinase domain (KD). Ask1 activity is attenuated by Akt Ser83 phosphorylation in mammals. Red arrows are used for inhibitory events. Green arrows for activator interactions.

5.3 Ask1 in damage response

Ask1 is required for oxidative stress-induced cell death in different contexts (Tobiume *et al.*, 2001). UV irradiation (Van Laethem *et al.*, 2006)), different cytotoxic stressors such as methylglyoxal (Du *et al.*, 2001), metal ions (Hansen, Zhang and Jones, 2006) and

anticancer drugs (Saeki *et al.*, 2002) have been shown to trigger apoptosis in part through ROS-mediated Ask1 activation. Furthermore, Ask1 contributes to endoplasmic reticulum (ER) stress-mediated cell death (Nishitoh *et al.*, 2002).

5.4 *Drosophila* Ask1

Drosophila has a single ortholog of Ask1 that presents two different isoforms of different length: Ask1-RC and Ask1-RB. In this animal model it has been also identified this apoptosis-inducing activity of Ask1. Using genetic analyses Ask1 and a *Drosophila* ortholog of TRAF (DTRAF1) have been identified as mediators of cell death induced by the expression of *rpr* (Kuranaga *et al.*, 2002). Overexpression of DTRAF1 activated Ask1 and induced JNK-dependent cell death. On the other hand, mutation of DTRAF1 or expression of dominant negative mutants of Ask1 or JNK suppresses *rpr*-induced cell death, suggesting that TRAF-Ask1-JNK pathway mediates conserved death signaling.

6. p38 signaling pathway

6.1 p38 MAPKs family.

The family of p38 MAPKs comprises 4 members. p38 alpha was the first to be identified by four different groups independently as a 38 kDa protein kinase that was rapidly phosphorylated on tyrosine (Tyr) in response to different stressors (Freshney *et al.*, 1994; Han *et al.*, 1994; Lee *et al.*, 1994; Rouse *et al.*, 1994). The other four family members were subsequently cloned and named *p38 beta*, *p38 gamma* and *p38 delta* and are approx. 60% identical in their amino acid sequence to p38 alpha (Jiang *et al.*, 1996, 1997; Lechner *et al.*, 1996; Mertens, Craxton and Goedert, 1996; Goedert *et al.*, 1997; Enslen, Raingeaud and Davis, 1998). The four p38 MAPKs are encoded by different genes and have different tissue expression patterns. Only *p38 alpha* is ubiquitously expressed at significant levels in most cell types. p38 MAPK pathway is strongly activated by stress and plays important roles in the regulation of cell survival, differentiation and in immune response, reviewed in (Nebreda and Porras, 2000; Ono and Han, 2000; Kyriakis and Avruch, 2001; Cuenda and Rousseau, 2007).

6.2 Activation of p38 by MAP2Ks and MAP3Ks.

p38 MAPKs are activated by dual phosphorylation in the activation loop sequence Thr-Gly-Tyr. This phosphorylation induces conformational changes that result in an open and extended conformation, ensuring substrate binding. In response to appropriate stimuli, Thr and Tyr residues can be phosphorylated by three dual-specificity MAP2Ks: MKK6, MKK3 and MKK4 (Enslen, Raingeaud and Davis, 1998; Alonso *et al.*, 2000). Several MAP3Ks have been shown to trigger p38 MAPK activation, among others, Ask1 and Transforming growth factor β -activated kinase 1 (TAK-1). Remarkably, some MAP3Ks that trigger p38 MAPK activation can also activate the JNK pathway (Cuevas, Abell and Johnson, 2007).

There are particular stimuli that trigger selectively the activation of different MAP3Ks. For instance, in *Drosophila* cells, MEKK1 activates p38 MAPKs in response to UV-irradiation or peptidoglycan exposition, whereas heat-shock activates p38 through

both Ask1 and MEKK1 (Zhuang *et al.*, 2006) In mammalian cells, oxidative stress results in p38 alpha activation by Ask1 (Dolado *et al.*, 2007).

6.3 Different levels of p38 activation

Strong activation of p38 MAPK is likely to result in an apoptotic response. On the other hand, low levels of p38 activity tend to be associated with cell survival (reviewed by (Dolado and Nebreda, 2008). Supporting these observations, continuous exposure to stresses, such as ROS, UV light or DNA-damaging agents induce cellular senescence. Interestingly, sustained activation of p38 MAPK by overexpression of a constitutively active MKK6 is sufficient to induce G₁ cell-cycle arrest with morphological and biochemical features of senescence (Haq *et al.*, 2002) .

6.4 Subcellular localization of p38

p38 alpha is detected in both the nucleus and the cytoplasm of non-stimulated cells. However, subcellular localization upon activation of p38 alpha is controversial. Some evidence points to translocation from the cytoplasm to the nucleus (Raingeaud *et al.*, 1995). One possible explanation for this change in subcellular localization is the involvement of p38 alpha in the regulation of G₂/M cell-cycle arrest to promote DNA repair. p38 alpha seems to translocate to the nucleus upon activation by different stressors that induce DNA damage, but not other stimuli (Wood *et al.*, 2009). In contrast, it has also been shown that in response to specific stimuli, p38 alpha preferentially accumulates in the cytosol (Ben-Levy *et al.*, 1998).

6.5 Interplay of p38 with other pathways

Evidences from cell lines and animal models' studies prove the interplay between the JNK and p38 MAPK pathways. Although some stimuli can activate simultaneously both pathways, JNK and p38 have antagonistic effects in many cases. The p38 MAPK pathway negatively regulates JNK activity at the level of MAP3Ks, either by phosphorylating the TAK-1 regulatory subunit TAB1 or MLK3 or, in some cases, p38 alpha also antagonize JNK by cell-type -specific mechanisms as reviewed in (Wagner and Nebreda, 2009).

There is also evidence of an interplay between Akt and p38 at different levels. p38 alpha may act as a negative modulator of Akt. Some evidence shows that p38 regulates the interaction between caveolin-1 and PP2A through a mechanism dependent on cell attachment, independently of PI3K (Zuluaga *et al.*, 2007). Also, it has been shown that silencing of p38 alpha inhibits Akt activation in mouse macrophages, a mechanism that seems to regulate macrophage apoptosis *in vivo* (Seimon *et al.*, 2009).

6.6 p38 pathway in *Drosophila*

In *Drosophila*, three different p38 MAPKs have been identified p38a, p38b and p38c. The MAP2K in charge of specifically activating p38 in *Drosophila* is Licorne (Lic) (Suzanne *et al.*, 1999). Some upstream kinases have been proposed to activate the p38 MAPK pathway, including MEKK1, TAK-1, Ask1 and Slipper (Slpr) (Fig. 7) (Inoue *et al.*, 2001; Chen *et al.*, 2010). It seems that the three family members of p38 MAPKs in *Drosophila* do not act redundantly. p38a regulates stress and the DUOX system in the midgut of flies, p38b seems to play a critical role in p38 signaling and p38c may have specific functions

in the intestine (Craig *et al.*, 2004; Seisenbacher, Hafen and Stocker, 2011; Vrila-Mortimer *et al.*, 2011).

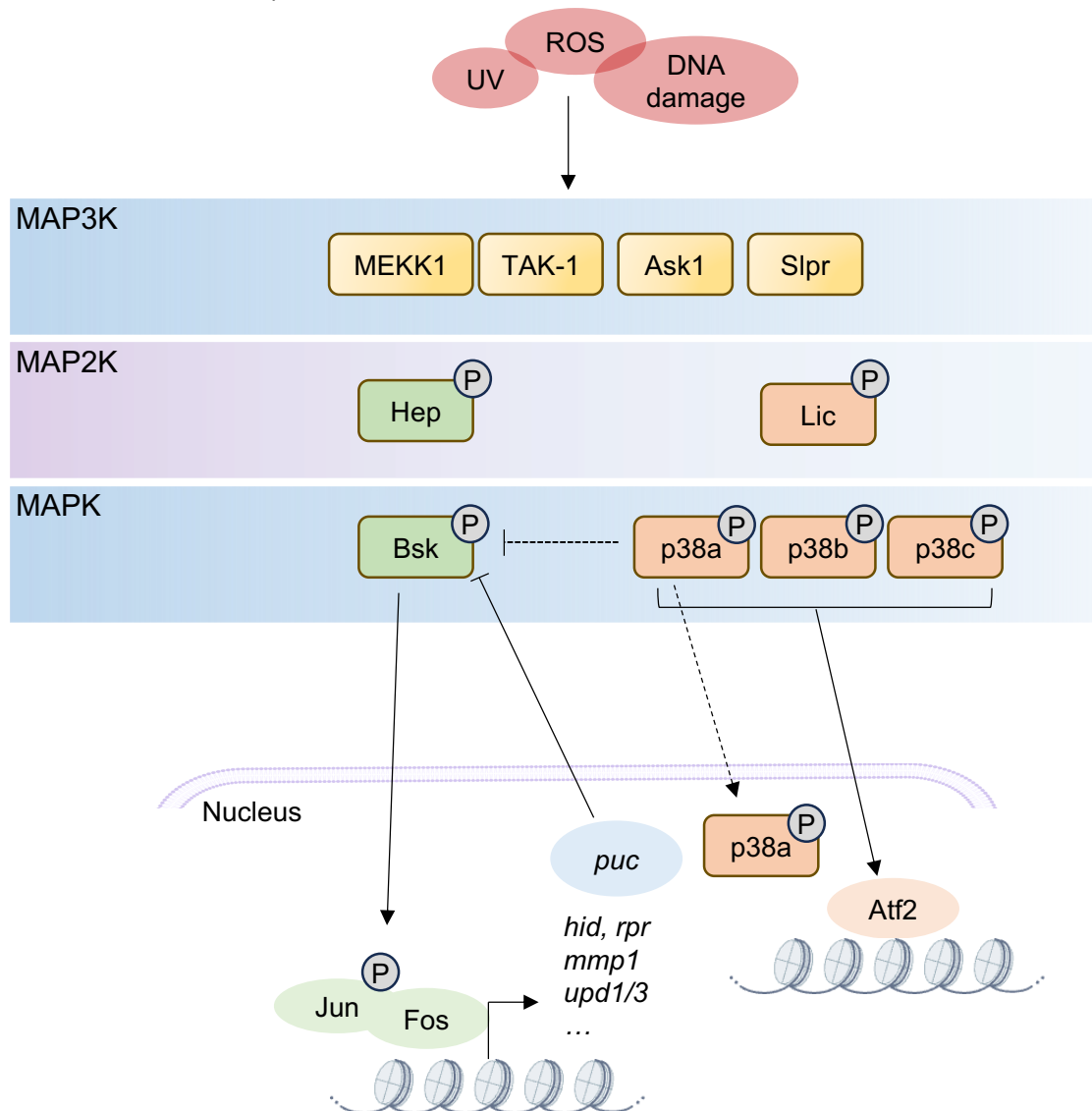


Fig. 7. SAPK in the p38 (orange) and JNK (green) pathways of *Drosophila*. After different stress stimulus (ROS, UV, DNA damage) appear, Hep is the mediator between *Drosophila* MAP3Ks and Bsk activation (the JNK fly homolog). Bsk phosphorylates the Jun TF triggering the association with Fos to form the AP-1 complex that goes into the nucleus and promotes transcriptional activation of many genes, among them *puckered* (*puc*), which encodes for a dual specificity phosphatase that turns off the activity of the JNK pathway. Lic, in the p38 pathway, mediates the activation of p38a,b,c downstream of MAP3Ks. Those kinases phosphorylate TFs as Atf2 (Activating TF2) (Tibbles and Woodgett, 1999), which goes to the nucleus and promote transcriptional response. p38a has been also described to enter into the nucleus upon activation.

6.7 p38 in imaginal disc regeneration and beyond

In *Drosophila* imaginal disc regeneration, it has been observed that after physical injury or genetic ablation ROS stimulates p38 phosphorylation. Incubation of injured discs with antioxidants (i.e., NAC) or ectopic activation of the enzymes in charge of detoxifying ROS: Sod1 and Cat, inhibits the activation of p38 upon injury.

Furthermore, p38 signaling seems to be required for tissue repair after genetic ablation as far as different mutant backgrounds or RNAi of the MAPK *p38* and also its

MAP2K *lic* impairs severely regeneration. p38 and JNK act independently during imaginal disc regeneration because mutant backgrounds for *hemipterous* (*hep*), the MAP2K of JNK in *Drosophila*, still being able to activate P-p38 near the wound after physical injury. Also, inhibition of JNK using drugs (JNK inhibitor IX) does not seem to affect the activation of p38 upon genetic ablation (Santabábara-Ruiz *et al.*, 2015). In *Drosophila* midgut it has been described that p38 signaling is activated in a ROS-dependent fashion in response to stresses including pathogenic bacterial infection and chemical/mechanical insult in the enterocytes. p38 signaling is required upon infection to promote intestinal stem cell (ISC) regeneration (Patel *et al.*, 2019).

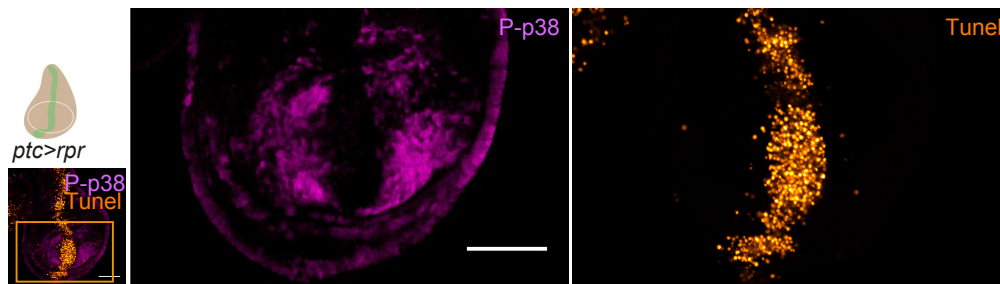


Fig. 8. p38 activation in imaginal discs after genetic ablation. Antibody against phospho-p38 was used to detect activation of the pathway upon cell death in the *ptc* region (depicted in green in the cartoon). In the zooms, active form of p38 is mostly detected in living cells surrounding the dying domain (dying cells detected by TUNEL staining (Orange)). There is no co-localization of P-p38 and dying cells. Scale bars=50µm.

7. JNK pathway

7.1 JNK MAPK family

The c-Jun NH2-terminal kinase (JNK) pathway is one of the MAPKs activated primarily by environmental stressors (Kyriakis and Avruch, 1990; Kyriakis *et al.*, 1994; Davis, 2000; Weston and Davis, 2002). In addition, some cytokines can operate as activators of the JNK pathway, such as $\text{TNF}\alpha$, interleukin-1 (IL-1) and growth factors (Ichijo, 1999).

JNK pathway is conserved in all metazoans regulating important physiological functions in cell proliferation, apoptosis, cell migration and regeneration (Zeke *et al.*, 2016; Pinal, Calleja and Morata, 2019). In mammals, there are 3 JNK genes: *Jnk1*, *Jnk2*, and *Jnk3*. Each three have alternative splicing forms, resulting in at least 10 different JNK proteins of 46–55 kDa identified. All 10 isoforms exert different substrate affinity (Dérillard *et al.*, 1994) and therefore, different JNK isoforms may play specific roles in multicellular organisms.

7.2 Activation of JNK by MAP2Ks and MAP3Ks and its substrates

The MAP2Ks MKK4 and MKK7 phosphorylate the Thr and Tyr residues within the activation loop motif (T183 and Y185 in JNK1) resulting in JNK activation. Fourteen different MAP3Ks activate the MKK4/MKK7-JNK pathway (Johnson and Nakamura, 2007). Several of these MAP3Ks are also capable of activating other MAP2Ks, for example, MKK3 and 6 for p38 pathway activation (Gallo and Johnson, 2002).

JNK interacts and phosphorylates the N-terminal transactivation domain of c-Jun enhancing its ability to activate related gene expression (Gupta *et al.*, 1995). JNK also phosphorylates a diversity of substrates, including other TFs and some non-nuclear proteins. JunB, JunD, c-Fos, and ATF are examples of TFs activated via JNK. One of them, c-Fos, together with c-Jun, constitute the Activator Protein-1 TF family (AP-1 family), which can regulate the expression of several stress-responsive genes (Yang *et al.*, 1998).

7.3 JNK pathway in *Drosophila*: apoptosis and regeneration

In *Drosophila*, there is a single JNK gene called *basket* (*bsk*) (*Bsk*). *Bsk* is a substrate of Hemipterous (*Hep*) MAP2K, (Igaki, 2009). The activation of *Bsk* results in the phosphorylation of the TFs Jun and Fos (AP-1) (Fig. 7) (Riesgo-Escovar and Hafen, 1997; Ciapponi *et al.*, 2001). This complex operates as a transcriptional regulator of a wide range of cellular functions.

In *Drosophila*, JNK pathway exerts different functions during development: (1) Dorsal closure during late embryogenesis (Glise, Bourbon and Noselli, 1995; Riesgo-Escovar *et al.*, 1996), and (2) fusion of left and right sides on the midline of the adult cuticle (Zeitlinger and Bohmann, 1999).

In addition, *Drosophila* JNK has a pivotal role in response to stressors such as ionizing irradiation and tissue damage, this has been observed particularly in imaginal discs (Bosch *et al.*, 2005; Pérez-Garijo, Shlevkov and Morata, 2009). JNK activity concentrates around damaged cells, which suggests that JNK activation upon stress is local (Fig 9). Moreover, this activity results in the expression of the JNK-dependent dual phosphatase *puckered* (*puc*) (Santabábara-Ruiz *et al.*, 2015) which in turn is an antagonist of the JNK activity (Martín-Blanco *et al.*, 1998).

Therefore, the activity of JNK is controlled by *puc* in order to avoid deleterious high levels of JNK signal (Fig 7). This is a key issue for the survival of cells as JNK is known to trigger apoptosis, and therefore JNK could propagate the elimination of cells beyond the damaged zone. Moreover, JNK creates an amplification loop of apoptosis as it triggers the apical caspase Dronc (Wells, Yoshida and Johnston, 2006). In conclusion, *puc* expression in cells near the damaged zone indicates that the tissue responds to damage by activating JNK but at the same time protecting it from propagating apoptosis by maintaining low levels of JNK activity. This, indicates that healthy cells protect themselves by prevention of scattering apoptosis by JNK-dependent *puc*.

Low levels of JNK can have another function in the expression of cytokines downstream the JAK/STAT pathway. Genetic ablation or physical injuries result in expression of the interleukin-6 (IL-6) family of cytokines Unpaired-1 (*Upd1*) and Unpaired-3 (*Upd3*) which are needed to trigger regeneration (Jiang *et al.*, 2009; Wu, Pastor-Pareja and Xu, 2010; Álvarez-Fernández *et al.*, 2015).

Thus, JNK could also have a pro-proliferative function in regeneration (Bosch *et al.*, 2005; Khan *et al.*, 2017; Martín, Pinal and Morata, 2017). The JNK reporter *TRE-DsRed.T4*, which monitors the AP-1 activity (Chatterjee and Bohmann, 2012), is detected in both apoptotic cells but also adjacent healthy cells. The reporter *puc-lacZ* (Martín-Blanco *et al.*, 1998) is expressed where the proliferative response takes place and rarely in the apoptotic cells (Bergantiños, Corominas and Serras, 2010). Furthermore, some *puc*

positive cells co-localize with EdU incorporating cells (proliferating cells), supporting the idea of JNK activation in living cells (Santabárbara-Ruiz *et al.*, 2015).

JNK role in regeneration is mediated downstream by Wg and JAK/STAT, known to act as growth factors (Mattila, Omelyanchuk and Nokkala, 2004; Jiang *et al.*, 2009; Smith-Bolton *et al.*, 2009; Katsuyama *et al.*, 2015). Both pathways are implicated in imaginal discs regeneration as well as other model organisms (ie., Planarias and Hydra) (Petersen and Reddien, 2008; Chera *et al.*, 2009; Smith-Bolton *et al.*, 2009; Santabárbara-Ruiz *et al.*, 2015).

We conclude that different levels of JNK activity operate during imaginal discs regeneration: (1) High levels in dying cells, to warrant apoptosis and (2) lower or transient in healthy cells surrounding the damaged area to ensure survival and proliferation (Fig. 9).

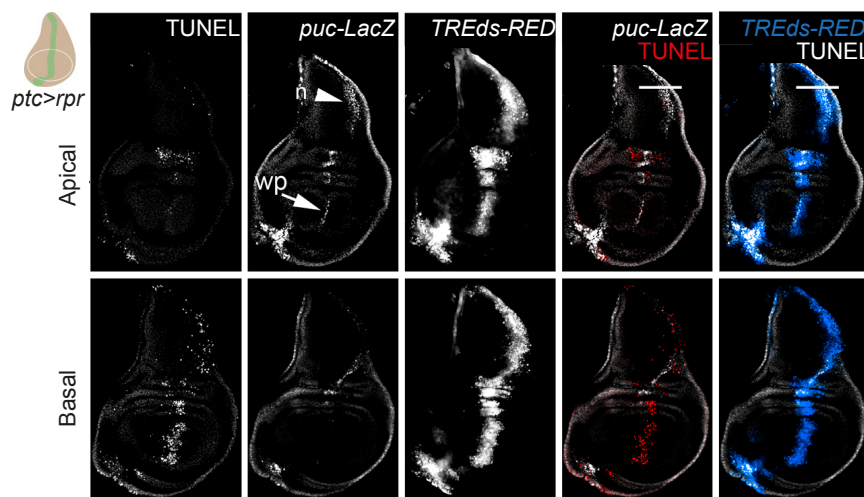


Fig.9. JNK activation in imaginal discs after genetic ablation. JNK activity reporters. All images correspond to the same disc after *ptc>rpr* induction (green zone in the cartoon). Top: apical sections of the imaginal disc. Bottom: basal sections. Note that *puc* is more abundant in apical than basal sections, particularly in the notum (n; arrowhead) and wing pouch (wp; arrow). Cell death (detected by TUNEL) and high TREDs-red are more abundant in basal sections, and clearly co-localize. Adapted from: (Santabárbara-Ruiz *et al.*, 2015).

8. PI3K/AKT pathway and Insulin signaling

One of the major effectors controlling tissue growth is the phosphatidylinositol 3-kinase PI3K/Akt pathway which is highly conserved throughout evolution (Brogiolo *et al.*, 2001; Engelman, Luo and Cantley, 2006). Insulin-like signaling controls systemic nutrient sensing and mediates its growth-regulatory function through PI3K/Akt pathway (Hietakangas and Cohen, 2009).

8.1 Nutrient sensing and control of development through PI3K/AKT pathway

Two main nutrient sensing pathways play pivotal roles in regulating growth control in *Drosophila*. The first pathway involves sensitive mechanistic Target Of Rapamycin (mTORC1), where nutrients trigger the activation of the mTORC1 complex. Subsequently, mTORC1 phosphorylates proteins such as 4EBP1 and S6k, thereby promoting translation processes crucial for cellular growth and proliferation (Zhang *et al.*, 2000). The second pathway, the PI3K/Akt pathway, responds to growth factors such as insulin. In *Drosophila*, a range of growth factors can stimulate the PI3K pathway, with prominent examples including insulin-like peptides (Ilps). These Ilps (Seecof and Dewhurst, 1974; Normann, 1975), akin to mammalian insulin, are synthesized and secreted by specialized cells known as insulin-producing cells (IPCs) located within the corpora cardiaca of the fly brain (Ikeya *et al.*, 2002; Rulifson, Kim and Nusse, 2002; Kim and Rulifson, 2004).

Upon release into the hemolymph, Ilps act as potent growth-promoting factors by binding to insulin-like receptor (InR) on the surface of target cells throughout the organism (Brogiolo *et al.*, 2001). Upon binding, the interaction between Ilps and InR triggers a signaling cascade that activates the PI3K pathway. This activation initiates a series of intricate intracellular events, culminating in the phosphorylation and activation of downstream effectors such as Akt. Akt, in turn, acts as a central regulator of cellular growth, metabolism, and survival (Verdu *et al.*, 1999; and reviewed in Manning and Cantley, 2007), orchestrating various processes essential for *Drosophila* development and physiology.

Furthermore, beyond Ilps, other growth factors and signaling molecules may also converge on the PI3K pathway in *Drosophila*, adding layers of complexity to the regulation of growth and metabolism in this model organism (reviewed in Hietakangas and Cohen, 2009).

In *Drosophila*, loss-of-function mutants affecting various components of the PI3K/Akt pathway have been observed to lead to either lethality or a noticeable reduction in body size (Böhni *et al.*, 1999). Conversely, mutations in the tumor suppressor Phosphatase and Tensin Homolog (Pten), an antagonist of PI3K, result in heightened Akt activity and subsequent tissue overgrowth (Goberdhan *et al.*, 1999; Huang *et al.*, 1999). These findings underscore the critical role of PI3K/Akt signaling in regulating growth processes within the organism. Given these observations, it is reasonable to hypothesize that the PI3K/Akt pathway plays a significant role in governing growth during regeneration. However, further investigation is warranted to elucidate the precise mechanisms by which this signaling pathway modulates regenerative growth in *Drosophila*. Of particular interest is understanding how nutrient availability or exposure to stressors, especially under conditions of starvation and exposure to ROS respectively, influence regenerative growth and how these environmental cues are sensed and transduced through the PI3K/Akt pathway.

Exploring these aspects could provide valuable insights into the intricate interplay between nutrient signaling, cellular growth regulation, and regenerative capacity in *Drosophila*, potentially offering parallels and novel perspectives for understanding regenerative processes in other organisms, including humans.

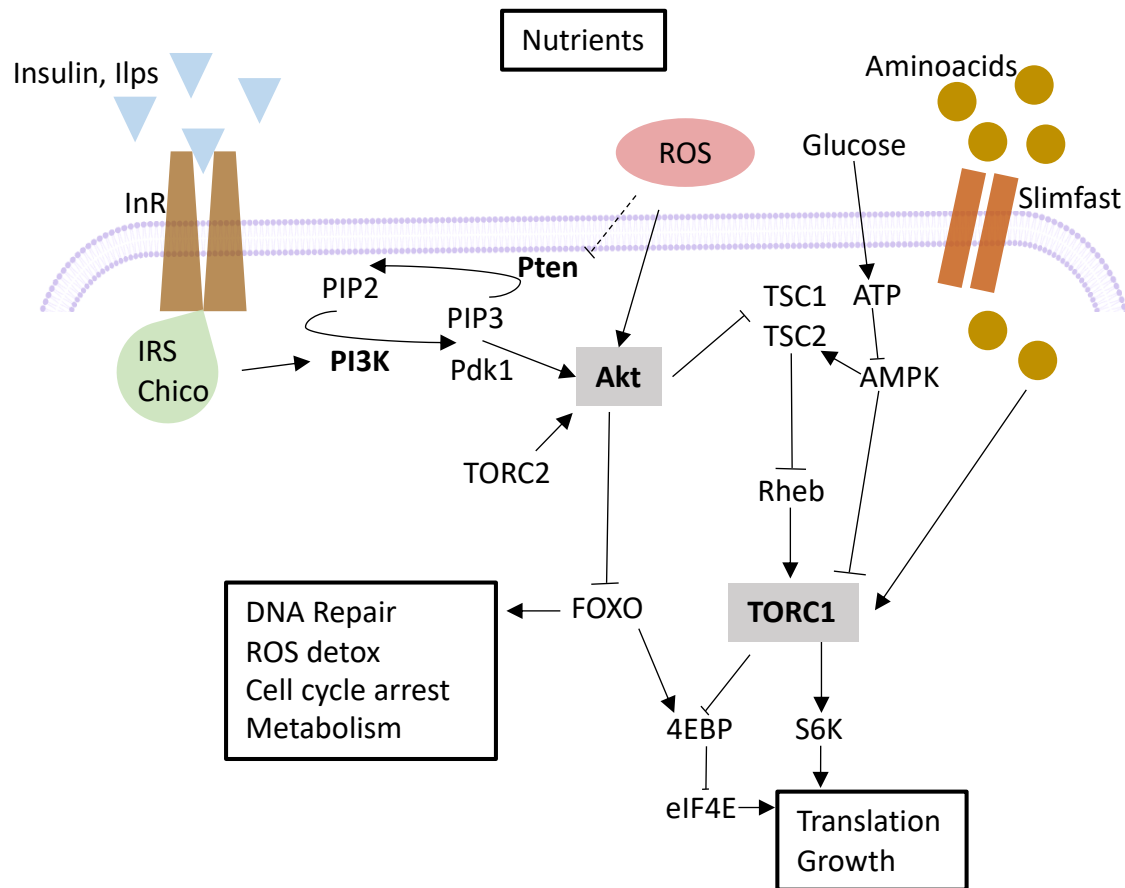


Fig. 10. The Insulin/IGF signaling (IIS) and mTORC1 signaling pathway in *Drosophila*. The IIS pathway shown at the left side of the cartoon, the TOR-signaling pathway is shown at right. Nutrition varies the release of Ilps. Ilps bind to InR which initiates a signal transduction cascade involving phosphorylation of intermediate proteins. Downstream growth effectors include FOXO, which is deactivated by IIS via phosphorylation by Akt. TOR also restricts the effects of dFOXO by inhibiting one of dFOXO's transcriptional targets, 4EBP. TOR is also regulated indirectly by IIS via the action of AKT on TSC1/2. TOR responds to aminoacids, and glucose, via the AMPK pathway. Both FOXO and TOR regulate the activity of multiple growth inhibitors and promoters, respectively.

In addition to these observations, it is worth mentioning the significance of Ilp8, a relaxin-like factor rather than an insulin-like peptide, in the systemic control of regenerative growth in *Drosophila*. Notably, upon injury to imaginal discs or genetic ablation, a delay in pupariation occurs (Hussey, Thompson and Calhoun, 1927; Simpson, Berreur and Berreur-Bonnenfant, 1980; Poodry and Woods, 1990; Smith-Bolton *et al.*, 2009; Halme, Cheng and Hariharan, 2010). This delay in development is provoked by the secretion of Ilp8 from imaginal discs into the hemolymph (Colombani, Andersen and Léopol, 2012; Garelli *et al.*, 2012). Moreover, Ilp8 expression is upregulated by certain signals detected within the imaginal disc during regeneration, such as activation of the JNK pathway (Colombani, Andersen and Léopol, 2012; Katsuyama *et al.*, 2015). Interestingly, Ilp8 exerts its modulatory effects on ecdysone production in the prothoracic gland by targeting the central nervous system (CNS), consequently delaying the onset of metamorphosis during regeneration (Colombani *et al.*, 2015; Garelli *et al.*, 2015; Vallejo *et al.*, 2015; Jaszczak *et al.*, 2016).

These findings underscore the intricate interplay between systemic and local signaling pathways during regenerative processes in *Drosophila*, highlighting the

multifaceted roles of Ilp8 in coordinating growth and developmental responses to injury or perturbation.

8.2 Fine tuning of PI3K/AKT pathway by ROS

The detection of signals during the early stages of regeneration, such as ROS, particularly H₂O₂, plays a crucial role in fine-tuning the insulin signaling pathway for optimal functioning (Lennicke and Cochemé, 2021b). Various phosphatases, including those that counteract the insulin-stimulated phosphorylation cascade, such as the tumor suppressor gene Pten, harbour critical Cys residues at their active sites susceptible to ROS-induced inactivation (Corcoran and Cotter, 2013). Similarly, several kinases involved in insulin signaling, such as Akt, are subject to redox regulation (Su *et al.*, 2019). As previously discussed, Akt, a pivotal kinase downstream of the PI3K/Akt pathway, phosphorylates Ser83 of human Ask1, suppressing the kinase activity of this MAP3K. Notably, Akt targets a consensus motif for recognition (RxRxx[ST]) located in the N-terminal region of human Ask1 (Kim *et al.*, 2001). This observation is intriguing as the attenuation of Ask1 could potentially result in moderate levels of JNK and p38 activation, which may be beneficial for tissue repair.

Of particular interest is whether the impact of ROS on the regulation of the PI3K/Akt pathway is relevant in *Drosophila* imaginal disc regeneration. Further analysis is warranted to determine whether *Drosophila* Ask1 contains a similar consensus motif for Akt recognition and to ascertain its relevance to the regeneration. Investigating these aspects could provide valuable insights into the intricate interplay between ROS signaling, the PI3K/Akt pathway, and tissue regeneration in *Drosophila*, potentially offering novel avenues for therapeutic intervention in regenerative medicine.

9. Tumor necrosis factor receptors (TNFR) signaling

TNF α is a proinflammatory cytokine with important functions in tissue repair, immunity, and cellular homeostasis (Pennica *et al.*, 1984; Beutler *et al.*, 1985). The mammalian TNF superfamily consists of 19 TNF-related ligands, each binding to one or more of the 29 TNFRs to trigger multiple processes from cell differentiation, proliferation to apoptosis, and innate and adaptive immune responses, reviewed in (Dostert *et al.*, 2019)

TNFR are transmembrane proteins that bind through its extracellular domain to the ligand, TNF α (Su, Dhusia and Wu, 2022). TNFRs initiate intracellular signaling cascades by recruiting adaptor molecules to their intracellular regions, including FADD/TRADD proteins and members of the TRAF family (Strasser, O'Connor and Dixit, 2000). TRAFs serve as adapter proteins, facilitating the activation of downstream signaling pathways (Dhillon *et al.*, 2019). Notably, TRAFs possess E3 ubiquitin ligase activity, with the exception of TRAF1, which lacks the ring domain. This ubiquitin ligase activity contributes significantly to their role in initiating and amplifying downstream signaling events. TRAFs have been identified as positive activator partners of MAP3Ks, crucially regulating the activation of MAPKs such as ERK-1, ERK-2, JNK, and p38 (Xia *et al.*, 2009; Shi and Sun, 2018). In the canonical classification of mammalian TNFRs, receptors are

categorized based on the presence or absence of a death domain (DD). Death receptors (DRs) possess a DD that binds adaptor proteins, subsequently recruiting TRAFs to initiate proapoptotic and proinflammatory signaling pathways (Dostert *et al.*, 2019). An exemplar of this is human TNFR1 (Brenner, Blaser and Mak, 2015).

Conversely, TNFR2, categorized as a non-death receptor lacking a DD domain, is activated by TNF α to promote tissue regeneration and cell survival (Faustman and Davis, 2010). The distinct signaling pathways initiated by TNFR1 and TNFR2 underscore the diverse physiological roles played by TNFRs in regulating cellular responses to TNF α stimulation. Further elucidation of the signaling mechanisms mediated by TNFRs holds significant promise for understanding their roles in health and disease, potentially informing therapeutic strategies targeting TNF α signaling pathways.

9.1 TNFRs in *Drosophila*

In contrast to this complex TNF-TNFR family in mammals, *Drosophila* TNF-TNFR system consists of just one single orthologue of TNF α , Egr, and two TNFRs, Wengen (Wgn) and Grindelwald (Grnd), providing an ideal genetic model for studying TNF-mediated processes *in vivo*. Wgn was initially identified as the first receptor for Egr in *Drosophila*, primarily based on its homology with other members of the TNFR superfamily and its ability to suppress *egr*-induced apoptosis (Kanda *et al.*, 2002; Kauppila *et al.*, 2003). This discovery suggested a crucial role for Wgn in mediating the apoptotic effects of Egr signaling. However, more recent research has cast doubt on the necessity of Wgn for *egr*-mediated apoptosis. Studies by Andersen *et al.*, have challenged the previous understanding by suggesting that Wgn may not be essential for the apoptotic functions of Egr (Andersen *et al.*, 2015).

In contrast, the other fly TNFR, Grnd, was identified in a genetic screen for suppressors of neoplastic growth (Andersen *et al.*, 2015). In this work the authors show that Grnd, and not Wgn, is required downstream of Egr to induce JNK-mediated apoptosis in both wing imaginal discs and the eye disc (Andersen *et al.*, 2015). Regarding the subcellular localization of both TNFRs in *Drosophila*, Grnd locates at the apical membrane while the majority of Wgn resides in intracellular vesicles. However, recent studies suggest that Wgn cycles between the plasma membrane, where it might engage with Egr, and intracellular compartments.

9.2 Biological functions of Grnd and Wgn

Although Grnd has not a DD, it employs DTRAF2 to promote JNK-dependent apoptosis through MAP3K TAK-1. It was also reported that the ectopic expression of Egr in discs results in its joint to Grnd and posterior internalization of the Egr-Grnd complex in vesicles. This seems to be key for *egr*-induced apoptosis (Igaki *et al.*, 2009; Ruan *et al.*, 2016). Immunostainings revealed that Wgn is located in vesicles, but never co-localizing with the Grnd-Egr membrane staining (Palmerini *et al.*, 2021). A recent work reveals that Grnd associates with Egr at nanomolar affinity level (KD around 30 nM), whereas Wgn presents low micromolar affinity for the ligand (KD in the order of 12 μ M) (Palmerini *et al.*, 2021). While this points to that Grnd and Wgn have nonredundant cellular functions, the role of Wgn in mediating Egr-dependent or independent processes in imaginal discs requires further investigation.

Interestingly, Wgn was described to trigger Egr-dependent functions in other tissues, where Grnd expression is very low or absent, this is the case in the gut and the brain. A common feature shared between all Wgn-mediated processes is that Egr comes from a proximal source (Palmerini *et al.*, 2021). While Egr recruit Grnd to mediate JNK-dependent apoptosis, its activation of Wgn results in cell survival behaviours and cytoprotective processes to fight against infections and promote tissue repair (Babcock, Landry and Galko, 2009; Andersen *et al.*, 2015; Jo *et al.*, 2017; Palmerini *et al.*, 2021; Loudhaief *et al.*, 2023). Also, Wng is found in many contexts to work in an Egr-independent manner: (1) in Photoreceptor axon targeting in neurons (Ruan *et al.*, 2013), (2) Tracheal branching in the embryo (Letizia, Espinàs and Llimargas, 2023) and (3) regulating lipid metabolism and immunity in the gut enterocytes (Loudhaief *et al.*, 2023). We think that the Wng-interacting molecules that initiates these Egr-independent functions deserve more attention. Moreover, it would be very interesting to further asses if Wng and Grnd have different/opposite roles in the imaginal disc regeneration process as well as to address if they are acting in an Egr-dependent or independent manner.

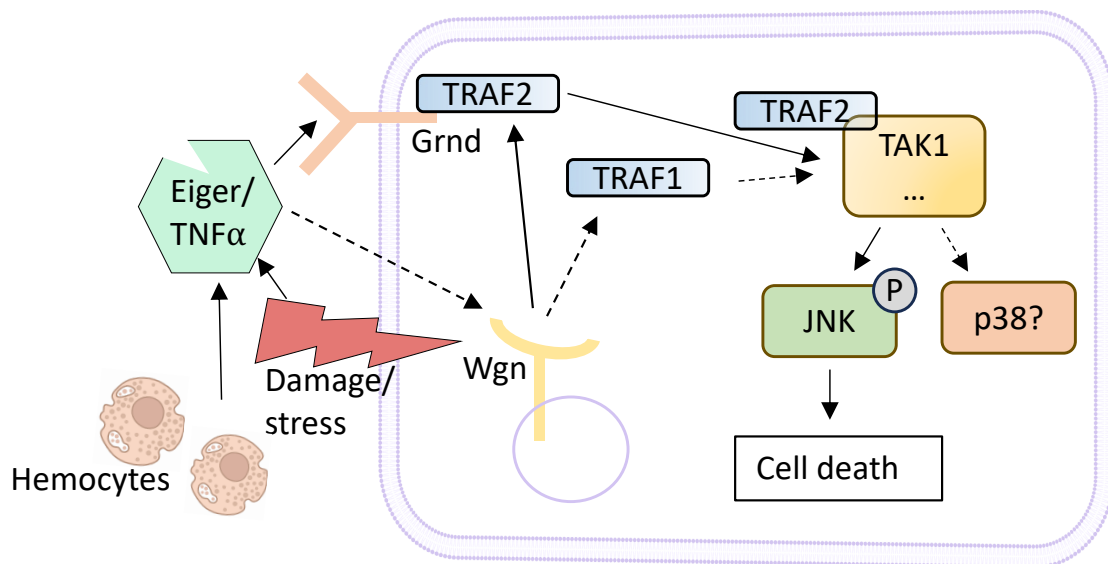


Fig. 11. Scheme of the Egr/TNFR pathway in *Drosophila*. Binding of Egr to Grnd triggers JNK pathway canonical activation via TRAF2 and activation of MAP3K TAK-1, resulting in cell death response. TNFR Wgn, located in vesicles, has ten orders of magnitude less affinity for Egr than Grnd and in many contexts can be activated in an Egr-independent manner. Wgn has been shown to interact with the different TRAFs described in *Drosophila*. In some tissues it has been also associated with a cell death response via JNK activation, but in many others Wgn is implicated in cell survival behaviors and cytoprotective processes to fight against infections and promote tissue repair.

9.3 The Cysteine-rich domain (CRD) of TNFRs

The aminoacidic sequence of TNFRs is poorly conserved throughout evolution except from the extracellular Cys-rich domain, which is the hallmark domain shared by the TNFR superfamily (Dominici *et al.*, 2004). The CRD is ~40 amino acids in length and contains 6 conserved Cys residues, which form a specific pattern of disulphide bridging (C1-C2, C3-C5, C4-C6) that provide structural rigidity to the domain. As explained before, Cys residues are ROS sensitive and it is widely accepted that oxidation of Cys residues in receptor proteins could result in its activation (Lipton *et al.*, 2002; Truong and Carroll,

2012). This raises an intriguing scenario that ROS produced by dying cells during the imaginal disc regeneration process might modulate the activation of Grnd and Wgn in *Drosophila*.

9.4 Evolution of TNFR in insects

Although TNFRs are found across all metazoan phyla (Wiens and Glenney, 2011), a comprehensive model of evolution elucidating the molecular origins of TNFRs in insects, including *Drosophila*, has remained elusive. Intriguingly, the death receptors (DRs) described above, are absent in choanoflagellates and Porifera, suggesting that the activation of cell death responses is not an ancestral characteristic of this protein family. It has been proposed that this role might have been acquired more recently in evolution, as DRs may have originated from a gene fusion event between proteins containing CRDs and those containing DDs in an ancestral planulozoan (Quistad and Traylor-Knowles, 2016)

To date, the evolutionary origins of *Drosophila* TNFRs and their relationship to TNFRs from other species have been poorly investigated, limiting our understanding of how the contrasting functions and molecular behavior of these two receptors may have originated. Further exploration into the evolutionary history of TNFRs in insects, including comparative genomic and phylogenetic analyses, is needed to shed light on the emergence and divergence of TNFR signaling pathways and their roles in diverse biological processes. This research could provide valuable insights into the evolutionary dynamics of cell death regulation and immune responses across different taxa, contributing to our understanding of the broader evolutionary landscape of TNFR-mediated signaling pathways.

Objectives

Objectives

Objectives

Main objective:

Metazoans are exposed to a wide variety of environmental stressors that can cause stress or damage, especially epithelial tissues which act as a first barrier in front of external insults. Understanding how early signals, such as ROS, released after trauma fine tune the response to damage in epithelia is an essential issue in regenerative biology.

Specific objectives:

Chapter 1

- Define how ROS impact MAP3K Ask1 activation resulting in different levels of SAPKs JNK and p38 activity.
- Decipher whether ROS, through Ask1 modulation, interacts with PI3K/Akt pathway controlling regeneration.

Chapter 2

- Study how Ask1 discriminate between p38 and JNK, fuelling p38 dependent regeneration.
- Determine if the nutritional status of *Drosophila* regulates p38 activation in cells involved in regenerative response via PI3K/Akt pathway.

Chapter 3

- Understand contrasting functions and molecular behaviour of TNFRs Wgn and Grnd in response to apoptosis.
- Describe how the TNF α /TNFR/TRAF axis contributes to apoptosis or survival in response to oxidative stress.
- Unveil the evolutionary origin of Wgn and Grnd and their relationship to TNFRs from deuterostome species

Publications



INFORME DEL DIRECTOR DE TESI DEL FACTOR D'IMPACTE DELS ARTICLES PUBLICATS. En cas que es presenti algun treball fet en coautoria, caldrà incloure també un INFORME DEL DIRECTOR de la tesi (signat), en què s'especifiqui exhaustivament quina ha estat la participació del doctorand/a en cada article i si algun dels coautors d'algun dels treballs presentats en la tesi doctoral ha utilitzat, implícitament o explícitament treballs per a l'elaboració de la tesi doctoral. Cal indicar factor d'impacte de l'any de publicació, quartil, decil i àrea de coneixement.

El Dr. **Florenci Serras i Rigalt**, director de la Tesi Doctoral elaborada per José Esteban Collado, amb el títol "Study of early signaling events governing epithelial regeneration"

INFORMA

Que la tesi doctoral s'ha elaborat com a un compendi de tres publicacions científiques, totes elles amb dades originals (articles 1-2-3 en el cos central de la tesi).

Article 1

Santabàrbara-Ruiz P, **Esteban-Collado J**, Pérez L, Viola G, Abril JF, Milán M, Corominas M, Serras F.

Ask1 and Akt act synergistically to promote ROS-dependent regeneration in *Drosophila*

PLoS Genet. 2019 Jan 24;15(1):e1007926. doi: 10.1371/journal.pgen.1007926.

Factor d'Impacte 2019: 5,17; Q1 en la categoria Genetics and Heredity.

Article 2

Esteban-Collado J, Corominas M, Serras F.

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

Development. 2021 Apr 15;148(8):dev197087. doi: 10.1242/dev.197087.

Factor d'Impacte 2021: 6,8; Q1 en la categoria Developmental Biology.

Article 3

Esteban-Collado J, Fernández-Mañas M, Fernández-Moreno M, Maeso I, Corominas M, and Serras F.

ROS-mediated TNFR Wengen activation in response to apoptosis

EMBO Journal (second revision)

Factor d'Impacte 2023: 11,4; Q1 en la categoria Biochemistry and Molecular Biology



A la primera publicació, que també es va presentar com a part de la tesi doctoral del Paula Santa Bárbara Ruiz, el doctorand va fer una part rellevant de les tasques experimentals. Concretament va estudiar la funció de Ask1 en l'activació de p38 durant la regeneració i estrès oxidatiu, un dels pilars d'aquesta publicació que ja porta 46 cites. A la segona publicació, el doctorand va dur a terme totes les tasques experimentals i analítiques, a més de col·laborar en la redacció del primer esborrany del manuscrit. A la publicació tercera, el doctorand va realitzar la part més rellevant del treball experimental i analític i va redactar el primer esborrany del manuscrit.

Barcelona 26 Abril 2024

Dr. Florenci Serras i Rigalt

Chapter 1

*“It takes grace to remain kind in **cruel** situations”*

Rupi Kaur, Milk and honey, 2014

RESEARCH ARTICLE

Ask1 and Akt act synergistically to promote ROS-dependent regeneration in *Drosophila*

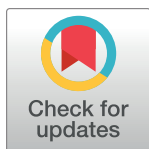
Paula Santabàrbara-Ruiz^{1☯}, José Esteban-Collado^{1☯}, Lidia Pérez², Giacomo Viola¹, Josep F. Abril¹, Marco Milán^{2,3}, Montserrat Corominas^{1,3}, Florenci Serras^{1*}

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Abstract

How cells communicate to initiate a regenerative response after damage has captivated scientists during the last few decades. It is known that one of the main signals emanating from injured cells is the Reactive Oxygen Species (ROS), which propagate to the surrounding tissue to trigger the replacement of the missing cells. However, the link between ROS production and the activation of regenerative signaling pathways is not yet fully understood. We describe here the non-autonomous ROS sensing mechanism by which living cells launch their regenerative program. To this aim, we used *Drosophila* imaginal discs as a model system due to its well-characterized regenerative ability after injury or cell death. We genetically-induced cell death and found that the Apoptosis signal-regulating kinase 1 (Ask1) is essential for regenerative growth. Ask1 senses ROS both in dying and living cells, but its activation is selectively attenuated in living cells by Akt1, the core kinase component of the insulin/insulin-like growth factor pathway. Akt1 phosphorylates Ask1 in a secondary site outside the kinase domain, which attenuates its activity. This modulation of Ask1 activity results in moderate levels of JNK signaling in the living tissue, as well as in activation of p38 signaling, both pathways required to turn on the regenerative response. Our findings demonstrate a non-autonomous activation of a ROS sensing mechanism by Ask1 and Akt1 to replace the missing tissue after damage. Collectively, these results provide the basis for understanding the molecular mechanism of communication between dying and living cells that triggers regeneration.

Author summary

One of the early events that occur after tissue damage consists on the production of Reactive Oxygen Species (ROS), that signal to the surrounding tissue to initiate wound healing and regeneration. Many signaling pathways, such as JNK and p38, respond to oxidative stress and are necessary for regenerative growth. As the link between ROS and

Competing interests: The authors have declared that no competing interests exist.

regenerative signaling is not well understood, we decided to explore the mechanism that underlies this process. To do that, we genetically induced cell death in specific areas of *Drosophila* wing imaginal discs and then studied the mechanism that drives living cells to replace the damage zone until it is completely regenerated. We found that the *Drosophila* Apoptosis signal-regulating kinase 1 (Ask1), a protein that is sensitive to oxidative stress, is a key player in this scenario. This protein acts as an intracellular sensor that upon damage activates the JNK and p38 regenerative signaling pathways. However, high activity of Ask1 can be toxic for the cell. This is controlled by Akt, a kinase downstream the insulin pathway, which attenuates the activity of Ask1 in the living cells that will participate in the regeneration process. In consequence, Ask1 and Akt act synergistically to respond to the stress generated after tissue damage and drive regeneration. Our results provide a first overview within the framework of how insulin signaling inputs could modulate the capacity to overcome tissue damage.

Introduction

Organisms are continuously exposed to a wide variety of environmental stressors that can cause deterioration and cell death. Tissues overcome the effect of those stressors by replacing damaged cells to restore homeostasis. Therefore, understanding the early signals that initiate the response to damage is an essential issue in regenerative medicine. Regeneration can be monitored in *Drosophila* imaginal discs, which are well-characterized epithelial sacs capable to regenerate after genetically-induced apoptosis or when parts are physically removed (reviewed in [1]). Compiling evidence supports that reactive oxygen species (ROS) fuel wound healing and oxygen-dependent redox-sensitive signaling processes involved in damage response [2–4]. Actually, genetically-induced apoptosis in the imaginal discs, using the Gal4/UAS system, leads to the production of ROS which propagate to the surrounding neighbors [5–7]. Although oxidative stress has been associated with several pathologies, it has been described that low levels of ROS can be beneficial for signal transduction [8].

The Jun-N Terminal kinase (JNK) and p38 signaling pathways are MAP kinases that respond to many stressors, including ROS, and foster regeneration and cytokine production in *Drosophila* [5,6,16–25,7,26,27,9–15]. Both pathways control numerous cellular processes as diverse as cell proliferation and cell death. For example, ectopic activation of JNK induces apoptosis [28,29], but its inhibition results in lethality [30]. It is thought that these disparities could be due to either different levels of activity or different mechanisms of activation.

After genetic ablation in a specific zone of the wing imaginal disc, dying cells produce high levels of ROS, and show high JNK activity [5,7]. High levels of JNK have been associated to dying cells and are involved in a feedback amplification loop to enhance the apoptotic response to stress [31]. However, the living cells nearby the dying zone show low levels of ROS, which are beneficial for the cell as they turn on the activation of p38 and low levels of JNK [5]. Both MAP kinases in the living cells are required for a cytokine-dependent regenerative growth. A key question is how the balance between the beneficial or detrimental effects of ROS is controlled and, in particular, how ROS control JNK and p38 activity. A candidate molecule to perform this function is the MAPKKK Apoptosis signal-regulating kinase 1 (Ask1), which responds to various stresses by phosphorylation of the kinases upstream JNK and p38 [32–34]. Hence, in a reduced environment, thioredoxin (Trx) inhibits Ask1 kinase activity by directly binding to the N-terminal region of Ask1. Upon oxidative stress, the redox-sensitive cysteines of Trx become oxidized, resulting in the dissociation of Trx from Ask1.

Consequently, Ask1 is oligomerized and its threonine-rich kinase domain is phosphorylated, inducing Ask1 activation [35,36]. Mammalian Ask1 is highly sensitive to oxidative stress and contributes substantially to JNK-dependent apoptosis. Nevertheless, recent studies have also revealed other functions of this kinase, including cell differentiation and survival [37].

Ask1-interacting proteins promote conformational changes that lead to the modulation of Ask1 activity and result in various cellular responses. For example, Ask1 is a substrate for phosphorylation by Akt1, a serine/threonine kinase activated by lipid kinase phosphatidylinositol 3-kinase (PI3K) pathway in response to insulin receptor activation. This phosphorylation is associated to a decrease of Ask1 activity *in vitro* [38]. The PI3K/Akt pathway, which is conserved between mammals and *Drosophila*, is one of the main effector signals for the regulation of tissue growth [39–41]. In *Drosophila*, loss-of-function mutants of various components of the pathway result in reduced body size or lethality [42]. Conversely, mutants of the phosphatase *PTEN*, an antagonist of PI3K, result in high activity of Akt and tissue overgrowth [43–45]. Thus, it is conceivable that PI3K/Akt signaling is involved in regenerative growth, but whether PI3K/Akt and Ask1 interact for controlling regeneration is unknown and deserves attention.

In this work, we genetically induced cell death in wing imaginal discs to explore the link between ROS production and regeneration. We found that Ask1 acts as a sensor of ROS upstream the JNK and p38 pathways. Moreover, we describe that Akt1 is necessary for modulating Ask1 activity in living cells to trigger regeneration. In addition, our results indicate that oxidative stress generated in the damaged cells signals the neighboring living cells to promote tissue repair.

Results

Ask1 is necessary for regenerative growth

The Gal4/UAS/Gal80^{TS} transactivation system has been extensively used to temporarily and spatially activate pro-apoptotic genes such as *reaper* (*rpr*) [10,11,46]. To study the capacity to regenerate, we induced cell death in the wing disc using the wing-specific *sal^{E/Pv}-Gal4* strain to activate *UAS-rpr*, in a temporally controlled manner thanks to the *tub-Gal80^{TS}* thermo sensitive Gal4-repressor (henceforth *sal^{E/Pv}>rpr*). We first kept the embryos at 17°C until the 8th day (192 hours after egg laying) to activate the *tub-Gal80^{TS}* and therefore to prevent *rpr* expression. Subsequently, we moved those larvae to 29°C for 11 hours, to inhibit *tub-Gal80^{TS}*, and activate apoptosis in the *sal^{E/Pv}-Gal4* zone. Then, larvae were returned to 17°C to avoid further apoptosis and allow tissue to regenerate. After adults emerged, wings were dissected and regeneration was analyzed. With these experimental conditions, 100% of the wings of *sal^{E/Pv}>rpr* flies contained the full set of veins and interveins, which demonstrates that the missing parts were regenerated (Fig 1A).

The same experiment was carried out in *Ask1* mutant backgrounds. The *Ask1^{MB06487}* mutant flies are viable in homozygosis but lethal over the deficiency *Df(3R)BSC636*, which suggests that is a hypomorphic allele. The *Ask1^{MI02915}* mutant is lethal in homozygosis as well as over the *Df(3R)BSC636*, which suggests that is an amorphic allele. We found that after genetically-induced cell death in *Ask1^{MB06487}* or *Ask1^{MI02915}* heterozygous background, full regeneration is only achieved in 15% and 23% of the wings, respectively (Fig 1A). The phenotypes of the abnormally regenerating wings vary and consisted in lack of some veins or parts of a vein or anomalous vein segments (S1A Fig).

To further address the role of *Ask1* in regeneration, we used a double transactivation system to simultaneously induce cell death in the *sal^{E/Pv}* domain and inhibit *Ask1* with *UAS-RNAi* (*UAS-Ask1^{RNAi}*) in an adjacent compartment (Fig 1B). RNAi knockdown of *Ask1* (*UAS-Ask1^{RNAi}*) reduces *Ask1* mRNA total levels to approximately 25–30 percent of that observed in controls

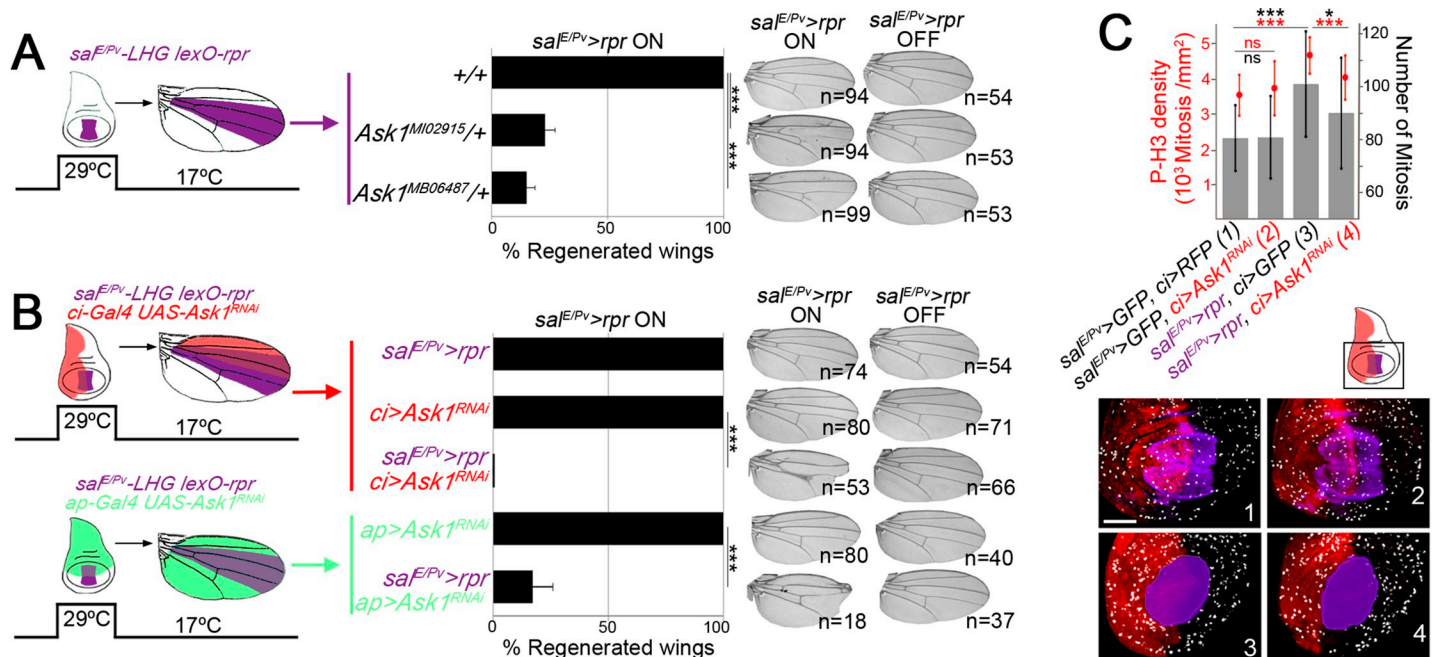


Fig 1. Ask1 is necessary for imaginal disc regeneration. (A) Scheme of the zone ablated (purple) in a wing imaginal disc and its corresponding region in the adult wing (left). Percentage of fully regenerated wings (normal set of veins and interveins) in wild type and *Ask1* mutant backgrounds after genetic ablation. On the right, examples of wings resulting after *rpr* activation (*sa^{E/Pv}>rpr* ON; developed at 17°C and for 11 hours at 29°C on day 8th after egg laying) and wings control, in the absence of cell death but with the same genotype (*sa^{E/Pv}>rpr* OFF, kept at 17°C from egg to adult) for the genotypes indicated. The number of cases analyzed in both conditions is indicated. (B) Double transgene activation scheme. The zone ablated in the wing disc using *sa^{E/Pv}-LHG lexO-rpr* and its corresponding region in the adult wing are indicated in purple; the zone of the *Ask1^{RNAi}* transgene expression is indicated in red or green. Percentage of normal wings (*Ask1^{RNAi}* expression alone), fully regenerated wings (*rpr* expression alone) and experimental samples (*rpr* and *Ask1^{RNAi}* dual expression). On the right, examples of resulting wings with *rpr* ON or OFF and the number of cases analyzed for each condition. (C) Phosphorylated H3 as indicator of mitotic cells per area (red) and number of mitosis (black) in discs with the dual transactivation system using *rpr* and *Ask1^{RNAi}* for the genotypes indicated (n = 30 for each genotype). The black square in the cartoon indicates the area used for analysis; below, examples of discs for the four genotypes used in this experiment. Note that the images have been colorized to maintain uniformity. Purple: GFP (1 and 2), cell death (3 and 4); Red: RFP (1), GFP (3), anti-Ci (2, 4). Mitosis (P-H3) in white. Bar: 50µm. Error bars in (A,B) show standard error of sample proportion and in (C) standard deviation. ***P<0.001.

<https://doi.org/10.1371/journal.pgen.1007926.g001>

(S1B Fig). The *UAS-Ask1^{RNAi}* transgene was activated in the anterior compartment using the Gal4/UAS system (*ci-Gal4>UAS-Ask1^{RNAi}*) and cell death was induced in the *sa^{E/Pv}* domain using the Gal80-repressible transactivator system *LHG* (*LexA-Hinge-Gal4* activation domain), a modified form of the *lexA lexO* system (*sa^{E/Pv}-LHG>lexO-rpr*) (Fig 1B) [5,47]. Both transgenes were activated at the same time, following the same protocol as in the previous experiment. The resulting adult wings (*ci>Ask1^{RNAi} sa^{E/Pv}>rpr*) lacked some veins or interveins and their size was reduced in all cases observed. However, the inhibition of *Ask1* in the anterior compartment for the same time but without cell death, did not affect vein pattern nor wing size (*ci>Ask1^{RNAi}*) (Fig 1B). Similar results were obtained after driving *UAS-Ask1^{RNAi}* to a different compartment (dorsal) and inducing cell death in *sa^{E/Pv}* (*ap>Ask1^{RNAi} sa^{E/Pv}>rpr*); in this case, we found that 82% of wings could not regenerate properly. As for the anterior compartment, *Ask1^{RNAi}* in the dorsal compartment without cell death did not affect vein pattern nor wing size (*ap>Ask1^{RNAi}*) (Fig 1B). To discard any toxicity due to the insertion of the transgene, all experiments were carried out in parallel but constantly at 17°C to maintain *tub-Gal80^{TS}* activity and block transgene (*UAS-* or *lexO-*) expression. In these conditions no defects in the wings were detected (*sa^{E/Pv}>OFF* in Fig 1A and 1B).

To evaluate whether these anomalies were due to impairment of proliferation, we analyzed the mitotic index, calculated as the number of cells positive for the phosphorylated form of

Histone 3 (P-H3) in the anterior compartment (*ci>*), where the *Ask1^{RNAi}* transgene was expressed. It is well known that apoptosis in discs induces compensatory proliferation of nearby cells (reviewed in [1]). Accordingly, genetic ablation (*sal^{E/Pv}>rpr ci>GFP*) resulted in an increase of mitosis compared to the neutral (*sal^{E/Pv}>GFP ci>RFP*) or to the *Ask1* knock-down (*sal^{E/Pv}>GFP ci>Ask1^{RNAi}*) conditions. However, combining genetic ablation in the *sal^{E/Pv}* zone with *Ask1* knockdown in the nearby anterior compartment, the number of mitosis associated to damage did not increase (*sal^{E/Pv}>rpr ci>Ask1^{RNAi}*) (Fig 1C). This observation confirms that *Ask1* reduction impairs regenerative growth.

Ask1 activity is higher in apoptotic cells than in regenerating cells

The *Drosophila Ask1* locus encodes two protein isoforms of different lengths, Ask1-RB and Ask1-RC, with predicted molecular weights of 136.5 kDa and 155.5 kDa, respectively. Both have a protein kinase-like domain that contains a highly conserved core of threonines conferring functionality to the protein (reviewed in [32]). It has been reported that after Trx release, the Ask1 oligomer undergoes conformational change leading to trans-autophosphorylation of the threonine residue corresponding to Thr838, Thr845 and Thr747 in human, mouse and *Drosophila Ask1*, respectively [32]. In human cells, phosphorylation of the N-terminus Ser83 by Akt results in attenuation of Ask1 activity *in vitro* [38]. This is noteworthy, because attenuation of Ask1 could result in moderate levels of activity necessary for turning on JNK and p38 in living cells. *Drosophila Ask1* lacks this residue and the Akt consensus motif in the N-terminus (S2 Fig, S1 Appendix, S2 Appendix). However, we found that the longer Ask1-RC isoform contained a conserved domain of unknown function DUF4071 with a highly conserved Ser (human Ser174, *Drosophila Ser83*), which we hypothesized that could be key in Ask1 regulation (Fig 2A, S2 Fig).

Active Ask1 can be traced with antibodies against the phosphorylated threonine residues of the highly conserved kinase domain (henceforth P-Thr) [48] and the attenuated form with specific antibodies against phosphorylated Ser83 (P-Ser83). We first tested both antibodies in wild-type wing imaginal discs. We detected low levels of P-Thr all over the disc including some transient high activity during mitosis (Fig 2B and S3A Fig), as occurs in dividing mammalian cells [49]. In *Ask1* mutant backgrounds, both the general and the mitosis-associated P-Thr levels were reduced but not abolished (S3B Fig), and *Ask1^{RNAi}* discs did not show any effect on P-Thr levels in mitosis (Fig 2E). This discrepancy may be due to the hypomorphic condition of the mutant combinations, but also to that the antibody may recognize some unspecific epitope in mitotic cells. We also found basal levels of P-Ser83 in wild-type discs (Fig 2C). Ectopic expression of *Ask1^{RNAi}* suppressed the low endogenous P-Ser83 Ask1 levels (S3C Fig).

Remarkably, upon apoptosis, high levels of P-Thr were localized in dying cells (Fig 2D) and absent or, in some cases, increased weakly above the basal levels in nearby living cells. This increase of P-Thr in apoptotic cells was inhibited after *Ask1^{RNAi}* expression (Fig 2E). In contrast, P-Ser83 accumulated in living cells adjoining the apoptotic zone and was completely absent in apoptotic cells. The increase in P-Ser83 varied from discs with strong accumulation near the dying domain to those with an extended increase in the whole wing pouch (2D and S4A Figs). Similar results were obtained after killing cells with a different pro-apoptotic gene (*sal^{E/Pv}>hid*) (S4B Fig). In the presence of apoptosis and blocking *Ask1*, the P-Ser83 increase in living cells was inhibited (*sal^{E/Pv}>rpr ci>Ask1^{RNAi}*, Fig 2F). Moreover, P-Ser83 was also found to be elevated at the wound edges after physical injury (S4C Fig). Together, these observations indicate that living cells respond to damage by phosphorylation of Ask1 Ser83.

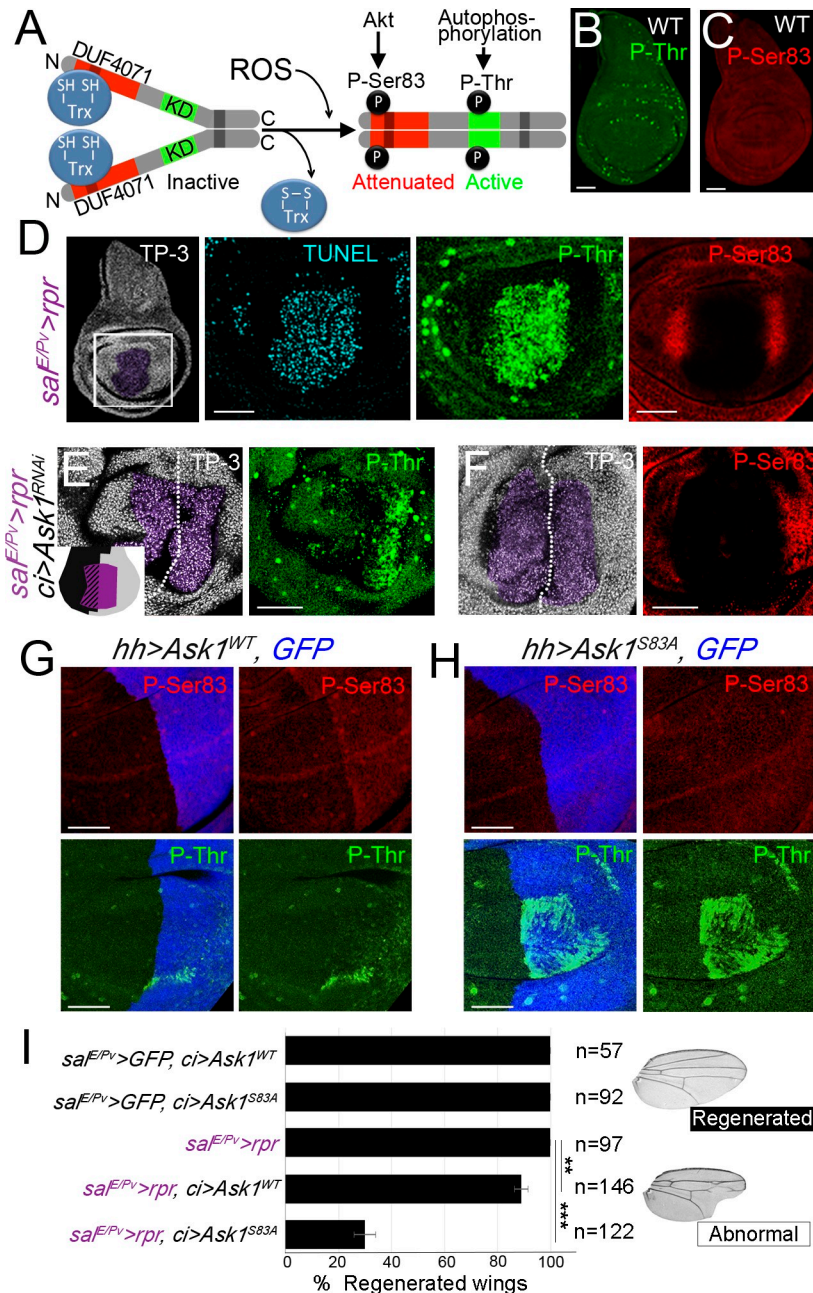


Fig 2. Ask1 activation in apoptotic cells and in regenerating cells. (A) Structural features of Ask1-RC isoform. The phosphokinase domain (KD) with a highly conserved core of threonines is shown in green. The DUF4071 domain, close to the N-terminal is shown in red. Inactive Ask1 is constituted by association of Ask1 monomers through the coiled coil domain (grey shadow) and the reduced form of thioredoxin (Trx-SH) in the N-terminal domain (left). Upon oxidation, Trx-S-S dissociates, which allows Ask1 autophosphorylation in the phosphokinase domain, leading to an active Ask1. The C-terminal coiled coil domain (dark grey) allows interactions between Ask1 monomers to form oligomers. Active Akt phosphorylates Ask1 on Ser83 residue. (B-C) Characterization of mammalian P-ASK1 antibodies in *Drosophila* discs. Endogenous levels of P-Thr (B) and P-Ser83 (C) in wild-type (WT) discs. (D) Ask1 activity in *sal^{EPV}>rpr* discs. *sal^{EPV}>rpr* disc indicating the area imaged (white square); TP-3 (TO-PRO-3) nuclei; purple area: the *sal^{EPV}>rpr* ablated zone. Disc stained with TUNEL assay and Ask1 P-Thr, (n = 21). Disc stained with Ask1 P-Ser83 (n = 35). (E-F) After cell death (purple) and simultaneous inhibition of Ask1 in the anterior compartment (*sal^{EPV}>rpr ci>Ask1^{RNAi}*), P-Thr (E) and P-Ser83 (F). White-dotted line indicates the AP boundary; anterior to the left, posterior to the right. The inset in (E) depicts the *Ask1^{RNAi}* area (black) and the *sal^{EPV}>rpr* ablated zone (purple). (G) Ectopic expression of the *Ask1^{WT}* form in the posterior compartment (blue). (H) Ectopic expression of the mutant form *Ask1^{S83A}*. Scale bars 50μm. (I) Percentage of regenerated wings in controls (*rpr*, *Ask1^{WT}* or *Ask1^{S83A}*) and *sal^{EPV}>rpr* discs. n=57, n=92, n=97, n=146, n=122. Regenerated wings are shown in black, abnormal wings in grey.

Ask1^{S83A} expression alone) and experimental samples (*rpr* and *Ask1^{WT}* or *Ask1^{S83A}* dual expression). Right: Examples of a wing with normal and abnormal regeneration (*sal^{E/Pv}>rpr*, *ci> Ask1^{S83A}*). Error bars show standard error of sample proportion. ***P*<0.01 ****P*<0.001.

<https://doi.org/10.1371/journal.pgen.1007926.g002>

In contrast to the high activity of Ask1 in dying cells (high P-Thr), the presence of P-Ser83 in living cells could be indicative of tolerable levels of Ask1 achieved by attenuation of P-Thr activity. To test this hypothesis, we mutated *Ask1* at serine 83 to alanine and cloned it into a UAS vector (*UAS-Ask1^{S83A}*). In parallel, we also cloned a wild-type form of *Ask1* (*UAS-Ask1^{WT}*). We ectopically expressed *Ask1^{WT}* in the posterior compartment of the wing disc (*hh-Gal4 UAS-Ask1^{WT}*), which resulted in an increase of P-Ser83, in addition to low levels of P-Thr (Fig 2G). Interestingly, the ectopic expression of *Ask1^{S83A}* in the posterior compartment (*hh-Gal4 UAS-Ask1^{S83A}*), did not show a rise in P-Ser83, but strong activation of P-Thr (Fig 2H). The high levels of P-Thr were not homogeneously distributed, varied from disc to disc, and were always found within the posterior compartment (*hh>*), where the transgene was activated. This observation concurs with the P-Ser83 residue as responsible for the attenuation of P-Thr.

We next analyzed whether the Ser83 residue was key for the damage response. Using the double transactivation system, we found that the expression of *UAS-Ask1^{WT}* or *UAS-Ask1^{S83A}* transgenes in the anterior compartment without cell death did not cause any defects in wing morphology, size or vein and intervein patterning (*sal^{E/Pv}>GFP ci>Ask1^{WT}* or *Ask1^{S83A}* in Fig 2I). After genetic ablation, expression of *UAS-Ask1^{WT}* resulted in full regeneration in 89% of wings (*sal^{E/Pv}>rpr ci>Ask1^{WT}*). In contrast, expression of *UAS-Ask1^{S83A}* led to a fall to only 29% of individuals being capable of regenerate and the rest showed strong effects on wing morphology, with altered pattern of veins and interveins, as well as the appearance of notches, which together are indicative of disrupted regeneration (*sal^{E/Pv}>rpr ci>Ask1^{S83A}* in Fig 2I). The suppression of the ability to regenerate by *UAS-Ask1^{S83A}*, which may act as a dominant negative allele, is likely due to the lack of non-autonomous P-Ser83 increase in the living cells near the damaged zone.

Pi3K/Akt signaling attenuates Ask1 activity in living cells

Next, we decided to identify the upstream signal responsible for Ser83 phosphorylation. In mammalian cells, attenuating phosphorylation of Ask1 is driven by the serine-threonine Akt kinase, the core kinase of the insulin pathway [38]. We wondered whether the *Drosophila* Akt1 as well as its upstream Pi3K92E kinase (the *Drosophila* Pi3K kinase also known as dp110), were required for Ser83 phosphorylation.

We first found that the active phosphorylated form of Akt (P-Akt) was increased in the living tissue and decreased or was absent in the dying zone (Fig 3A–3D). To test whether the Akt1 phosphorylated the endogenous Ask1 Ser83 *in vivo*, we specifically inhibited this kinase with a *UAS-Akt^{RNAi}* in a stripe of cells at the center of the disc, the *ptc* domain (*ptc>Akt^{RNAi}*), and found that P-Ser83 was reduced (Fig 3E and 3F). In addition, ectopic activation of Akt1 (*UAS-myr-Akt1.S*), a constitutively activated membrane-anchored form of *Akt1* [50], resulted in an increase in the levels of P-Ser83 (Fig 3G and 3H).

We next studied the role of P-Akt on Ask1 in the context of regeneration. In the presence of cell death and blocking *Pi3K92E*, with the dominant negative form *dp110^{DN}* in the adjacent compartment, we observed a reduction in the accumulation of P-Akt and P-Ser83 induced after genetic ablation (*sal^{E/Pv}>rpr hh> dp110^{DN}* in Fig 3I–3K). Moreover, in the overlapping zone of dying cells and *dp110^{DN}*, the accumulation of P-Thr was unaffected (Fig 3L and S5 Fig). We also scored the effects on wing regeneration after blocking *Akt* and *Pi3K92E* kinases.

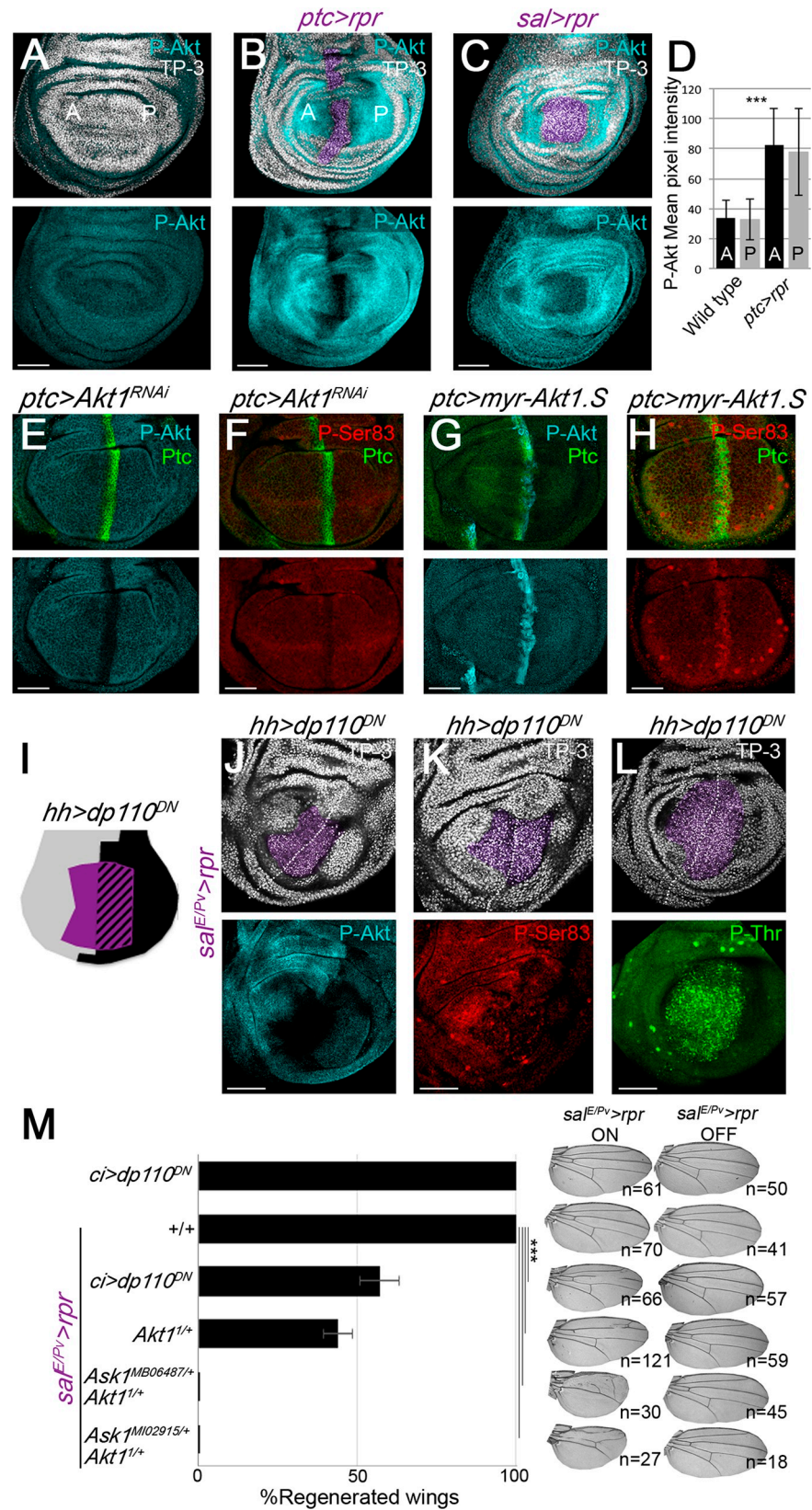


Fig 3. Phosphorylation of *Drosophila* Ask1 Ser83 depends on the Pi3K/Akt1 pathway. (A–C) P-Akt staining in wild-type discs (A), and after *ptc>rpr* (B), and *sal>rpr* induction (C). Apoptotic zone (pyknotic nuclei) in purple. (D) Mean pixel intensity for the wild type and *ptc>rpr* taken from regions of interest in the A and P compartments as shown in the images. (E,F) RNA interference of *Akt1* in the *ptc* stripe of cells (*ptc>Akt1^{RNAi}*) and (G,H) ectopic expression of a constitutively active form of *Akt1* (*ptc>myr-Akt1.S*), stained with P-Akt and Ask1 P-Ser83. (I) Design of the experiments in J,K and L with *sal^{E/Pv}>rpr* induction (purple) and blocking Pi3K (*hh>dp110^{DN}*) in the posterior compartment (black). Staining with anti-P-Akt (J), with anti-Ask1 P-Ser83 (K) with Ask1 P-Thr (L). White-dotted line indicates anterior-posterior boundary. TP-3; TO-PRO-3 nuclear staining. Scale bars 50µm. (M) Percentage of fully regenerated wings after genetic ablation and Pi3K pathway inhibition alone or in combination with *Ask1* mutant backgrounds (genotypes indicated). Right: *sal^{E/Pv}>rpr* ON, examples of wings with full regeneration (controls *sal^{E/Pv}>rpr* or *ci>dp110^{DN}* alone) and anomalous regeneration (*sal^{E/Pv}>rpr* with the genotypes indicated); *sal^{E/Pv}>rpr* OFF wings: examples of control wings in the absence of *rpr*-ablation (maintained at 17°C). Error bars indicate standard error of sample proportion. ****P*<0.001.

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Regeneration was impaired when the dominant negative form of *Pi3K92E* was expressed in the anterior compartment (*sal^{E/Pv}>rpr ci>dp110^{DN}*) as well as in the *Akt1¹* heterozygous background (*sal^{E/Pv}>rpr Akt^{1/+}*), an allele that encodes a catalytically inactive protein [51] (Fig 3M). Moreover, regeneration was severely affected in double heterozygous flies containing *Akt1¹* and *Ask1^{MB06487}* or *Ask1^{MI02915}* alleles (Fig 3M). Neither the dominant negative form of *Pi3K92E* nor the allelic combination *Akt1¹* and *Ask1^{MB06487}* or *Ask1^{MI02915}* affected wing development in the absence of cell death (*sal^{E/Pv}>OFF* wings in Fig 3M). These results further support the notion that *Pi3K92E/Akt1* and *Ask1* genetically interact and that their epistatic interaction is key to drive regeneration.

ROS promote Ask1 activation

Next, we modulated the ROS levels in order to determine if *Ask1* can sense oxidative stress after damage. We fed *sal^{E/Pv}>rpr* larvae with food supplemented with N-acetyl cysteine (NAC), a potent non-enzymatic scavenger that decreases ROS production, and examined *Ask1* phosphorylation (Fig 4A). After induction of cell death, we found a significant decrease in both P-Thr and P-Ser83 in discs from NAC-fed larvae (Fig 4B and 4C; S3 Appendix). In addition, we fed larvae with H₂O₂-supplemented food and observed a significant increase in both P-Thr and P-Ser83 levels (Fig 4D–4F). This oxidative stress-induced increase was blocked in the hypomorphic *Ask1^{MB06487}* homozygous mutant (Fig 4E and 4F; S3 Appendix). It is known that in mammalian cells *Ask1* can also be activated by endoplasmic reticulum (ER) stress [52]. Feeding larvae with tunicamycin, an inhibitor of N-glycosylation in the ER that induces ER stress, led to an increase in *Ask1* activation, which was also blocked in *Ask1^{MB06487}* mutant discs (Fig 4E and 4F; S3 Appendix).

Furthermore, we examined whether *Akt1* activation in the living cells is targeted non-autonomously by ROS produced by the dying cells. To examine this hypothesis, we enzymatically blocked ROS production using ectopic expression of the ROS scavengers *Superoxide dismutase 1* and *Catalase* (*Sod1:Cat*) in the *rpr* ablated region (*sal^{E/Pv}>rpr*, *Sod1:Cat*) and monitored the pixel intensities of two adjacent zones in each disc, the anterior (A) and posterior (P) to the *sal^{E/Pv}* (Fig 4G). The activation of the *Sod1:Cat* transgene concomitantly with *sal^{E/Pv}>rpr* did not inhibit apoptosis and P-Thr was detected in the dying cells (S6 Fig). However, in these conditions, we found that P-Akt levels in neighboring cells were reduced in both A and P zones when *Sod1:Cat* was co-expressed with *rpr*. Accordingly, we found that the increase of P-Ser83 in neighboring cells was blocked when *Sod1:Cat* was co-expressed with *rpr* (Fig 4G; S3 Appendix). Together, these results demonstrate that the oxidative stress generated from the dying cells targets *Akt1* and *Ask1* in the neighboring undamaged tissue.

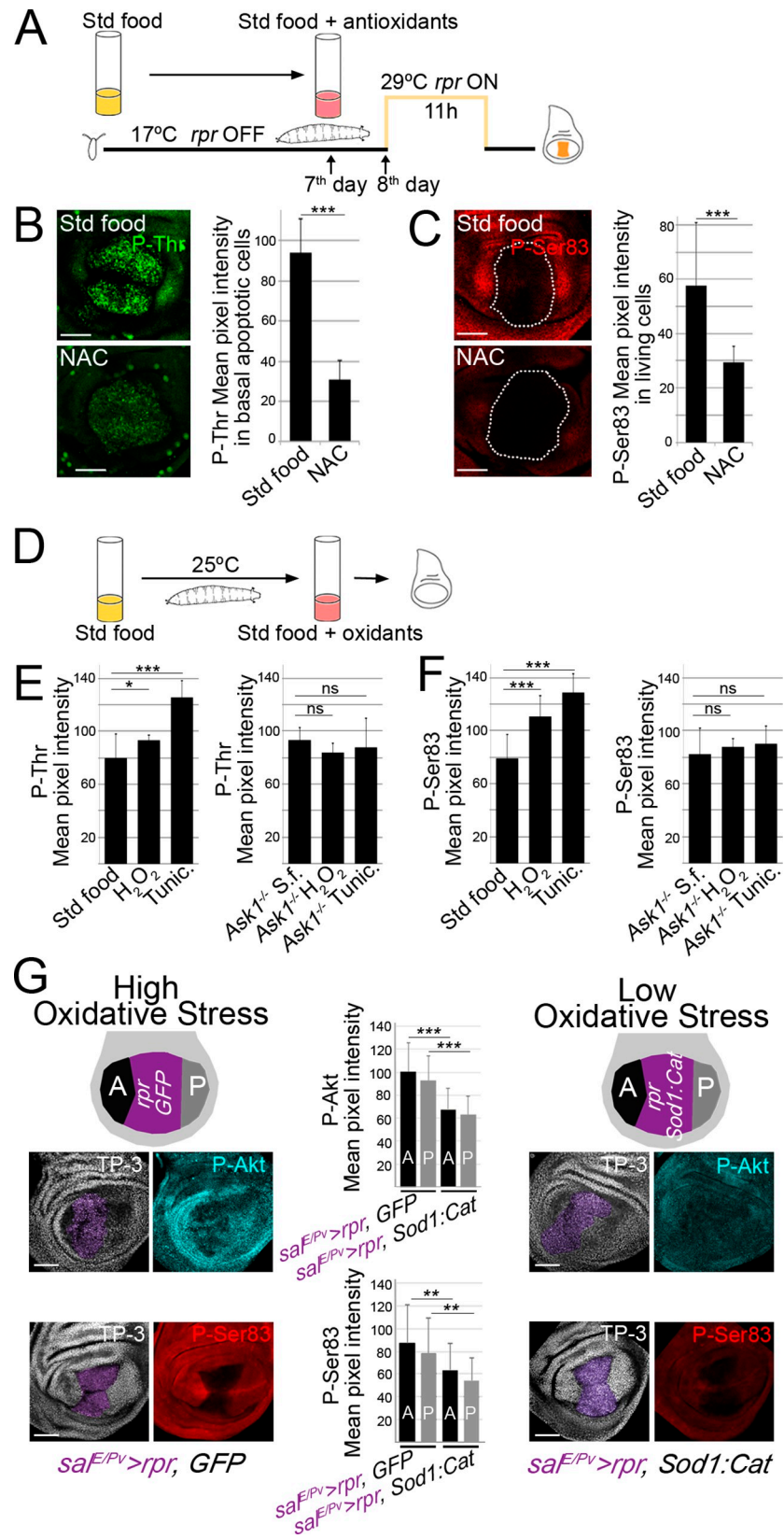


Fig 4. Ask1 is activated by ROS. (A) Design of experiments after feeding larvae with the NAC antioxidant. (B) Examples of Ask1 P-Thr after $sal^{E/Pv} > rpr$ induction in standard or NAC-supplemented food. Mean pixel intensities of P-Thr fluorescent labeling in the apoptotic zone of $sal^{E/Pv} > rpr$ discs from larvae fed with a standard or NAC-supplemented diet. (C) Examples of Ask1 P-Ser83 after $sal^{E/Pv} > rpr$ induction with standard or NAC-supplemented food. White dotted line indicates the $sal^{E/Pv} > rpr$ dying domain. Mean pixel intensities of Ask1 P-Ser83 fluorescent labeling in the neighboring cells near the apoptotic zone of $sal^{E/Pv} > rpr$ discs from larvae fed with a standard or NAC-supplemented diet. (D) Design of experiments after enhancing oxidative stress. (E) Left: P-Thr mean pixel intensity of wild-type wing discs from larvae fed with standard, H_2O_2 - and tunicamycin-supplemented food. Right: P-Thr mean pixel intensity of $Ask1^{MB06487}$ homozygous mutant discs from larvae fed with standard, H_2O_2 - and tunicamycin-supplemented food. (F) Left: P-Ser83 mean pixel intensity of wild-type wing discs from larvae fed with standard, H_2O_2 - and tunicamycin-supplemented food. Right: P-Ser83 mean pixel intensity of $Ask1^{MB06487}$ homozygous mutant discs from larvae fed with standard, H_2O_2 - and tunicamycin-supplemented food. (G) P-Akt (top) and P-Ser (bottom) mean pixel intensity from discs with rpr activation ($sal^{E/Pv} > rpr$, GFP; high oxidative stress) and from discs with simultaneous $Sod1:Cat$ and rpr activation ($sal^{E/Pv} > rpr$, $Sod1:Cat$; low oxidative stress). Purple area shows where transgenes were activated. A and P are the zones where pixel intensity was measured. S.f.: standard food. * $P < 0.05$. ** $P < 0.01$, *** $P < 0.001$. Error bars indicate standard deviation. TP-3: TO-PRO-3 nuclear staining. Scale bars: 50 μm .

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Ask1 activates p38 and JNK in regeneration

We have shown that the Ask1 P-Ser83 attenuating signal is essential for regeneration, and that this signal depends on ROS and Akt. We next hypothesized that this P-Ser83-mediated attenuation does not silence Ask1, but instead maintains the low levels of Ask1 activity necessary for regeneration. Therefore, we analyzed the expression of its known targets, the stress-activated MAP kinases JNK and p38 [48,53], both required for ROS-dependent regeneration [5,6]. We induced cell death in the $sal^{E/Pv}$ domain and simultaneously blocked Ask1 in the anterior compartment, and used the posterior as an internal control for the same disc ($ci > Ask1^{RNAi} sal^{E/Pv} > rpr$). We found most phosphorylated p38 (P-p38), as an indicator of p38 activity, in the posterior compartment (Fig 5A and 5B). The effects of Ask1 on JNK activity were monitored by matrix metalloproteinase 1 (Mmp1) expression as it is one of the bona fide transcriptionally regulated read-outs of the JNK pathway [54]. After $sal^{E/Pv} > rpr$ cell death, Mmp1 was found in both compartments, but in $ci > Ask1^{RNAi} sal^{E/Pv} > rpr$ discs Mmp1 mostly accumulated in the posterior (Fig 5C and 5D). The effects of Ask1 interference on JNK pathway were strengthened after staining with P-JNK antibody. P-JNK was found accumulated around the dead zone in $sal^{E/Pv} > rpr$ discs. However, when Ask1 was knockdown in the anterior compartment ($ci > Ask1^{RNAi} sal^{E/Pv} > rpr$ discs), P-JNK was reduced in this compartment and accumulated in the posterior (S7A and S7B Fig). To confirm that Ask1 is responsible for JNK and p38 activation, we tested whether Ask1 was required for their activation after physical damage. We made two incisions in $hh > GFP, Ask1^{RNAi}$ discs, one in the $UAS-Ask1^{RNAi}$ compartment and another into the control compartment. We found that P-p38 localized at the wound edges in the control compartment, whereas the levels of P-p38 were reduced at the wound edges of the $Ask1^{RNAi}$ compartment (Fig 5E and 5G). Likewise, cut $Ask1^{RNAi}$ discs resulted in less Mmp1 than in the control compartment (Fig 5F and 5H). In addition, we tested the *puckered* (*puc*) reporter of the JNK pathway, which normally accumulates in wounds, and found to drop in heterozygous Ask1 discs after a physical injury (S7C and S7D Fig). Together, these results indicate that Ask1 is necessary for the activation of p38 and JNK during regeneration.

Discussion

We have shown here that Ask1 acts as a sensor of ROS after damage, and that synergizes with Pi3K/Akt1 to phosphorylate the Ask1 Ser83, a key phosphorylation event to initiate regeneration. In addition, we have demonstrated that the activation of the Pi3K/Akt1 and

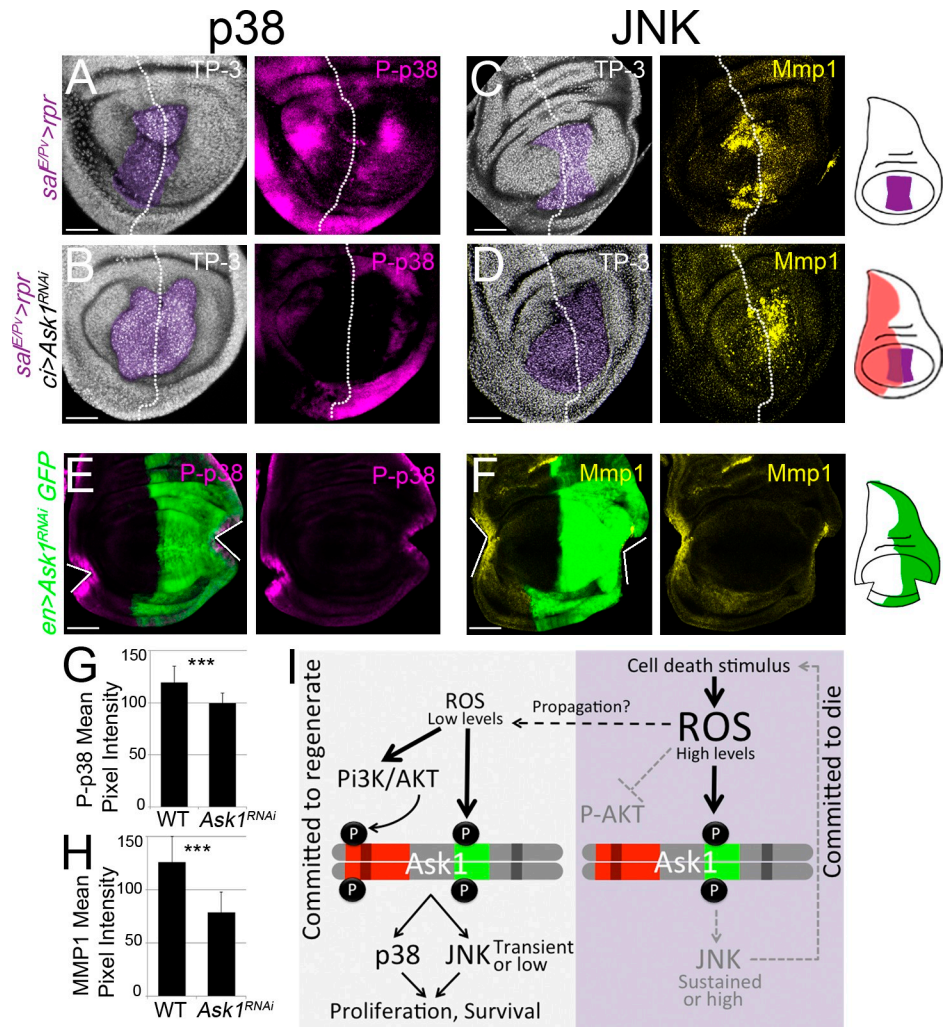


Fig 5. Ask1 promotes JNK and p38 signaling. (A) P-p38 after *sal^{E/Pv}>rpr* induction (n = 14). (B) Inhibition of *Ask1* in the anterior compartment (*sal^{E/Pv}>rpr ci>Ask1^{RNAi}*) stained for P-p38 (n = 7). (C) Mmp1 after *sal^{E/Pv}>rpr* induction (n = 16). (D) Inhibition of *Ask1* in the anterior compartment (*sal^{E/Pv}>rpr ci>Ask1^{RNAi}*) stained for Mmp1 (n = 14). TP-3: TO-PRO-3 nuclear staining. Purple: *sal^{E/Pv}>rpr* ablated zone. White dotted line divides the discs into anterior (*Ask1* inhibition; to the left) and posterior (wild-type; to the right) compartments. (E-H) The *UAS-Ask1^{RNAi}* was activated in the posterior compartment together with *UAS-GFP* (green). Two injuries were inflicted with tungsten needles in Schneider's medium, one in the anterior and one in the posterior compartment. Discs were stained for P-p38 and Mmp1. (E) For P-p38 staining, discs were immediately fixed after physical injury. (F) For Mmp1 staining, discs were first cut and cultured for 5 hours before fixation and processing. White lines indicate the wound site. Scale bar: 50µm. (G) Quantification of P-p38 activation at the wound site (n = 10 discs each genotype). (H) Quantification of Mmp1 at the wound site (n = 8 discs each genotype). Error bars indicate standard deviation. ***P<0.001. (I) Model proposed for the onset of regeneration in the columnar epithelium of the wing imaginal disc.

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Ask1 in undamaged cells is originated non-autonomously by ROS produced by the damaged tissue.

An essential question to understand regeneration is how damaged cells communicate to the nearby living tissue to initiate regenerative growth. One of the most classical and determining studies on epithelial regeneration was the discovery that massive cell death caused by ionizing radiation in *Drosophila* larvae resulted in proliferation of the unaffected neighbors to compensate the lost [55]. Since then, compensatory proliferation, a cellular response linked to

regeneration, has been considered as a result of signals released from apoptotic cells (reviewed in [56]). We propose here that the oxidative stress generated by damaged or apoptotic cells signals undamaged tissue to initiate the Ask1/Akt1 machinery that will culminate with repair and compensatory proliferation.

ROS acting in signaling after wounding has been subject of extensive research in various organisms and tissues [5,6,64–67,26,57–63]. Here, we have uncovered two scenarios of Ask1 activity: high activity in apoptotic cells, with high levels of P-Thr; and low activity in undamaged cells, where P-Ser83 prevails over P-Thr. This fits with the high levels of ROS and JNK in dying cells and the low levels of ROS, low JNK and P-p38 in living cells reported previously [5]. Together, our observations address the question of how JNK signaling selectively fosters apoptosis or proliferation, and how this signal is related to high or low ROS levels. The same applies to p38, which is only activated in unharmed cells when neighbors enter in apoptosis or are damaged [5,53].

Our study demonstrates that Ask1 operates in an Akt1-dependent manner in living cells and in an Akt1-independent manner in dying cells. It is conceivable that Akt1-mediated attenuation results in either low or transient levels of Ask1 activity necessary to stimulate P-p38 and low JNK levels for regeneration. In contrast, apoptotic cells induce high or sustained levels of JNK because high levels of ROS result in high Ask1 P-Thr in the absence of Akt1 attenuation.

Based on the results presented here and previous observations, we propose a model in which oxidative stress and the absence or presence of Pi3K/Akt1 survival signals are integrated in Ask1 to control the selective activity of JNK and p38, and in turn regenerative growth (Fig 5I). In this model, only dying cells produce high ROS levels and high JNK activity [5,7]. In addition, other mechanisms can exacerbate apoptosis through the lethal levels of JNK, and therefore increase ROS production. For example JNK, as well as p53, functions in a positive feedback after apoptosis as it transcriptionally activates the pro-apoptotic genes *hid* and *rpr*, thus amplifying apoptosis [31,68]. It is therefore likely that the high ROS that result in high Ask1 activation, inabilities the dying cells to respond to Pi3K/Akt1, preventing the Ask1 attenuation and resulting in deleterious JNK activity. We also have demonstrated that P-Akt1 is absent, or low, in dying cells, which supports a lack of Pi3K/Akt1-dependent Ask1 attenuated activity in dying cells. These cells will enter into a committed-to-die status with no return to survival. In contrast, the living neighbor cells, albeit adjacent to dying cells, show tolerable levels of ROS [5,7], and activate moderate levels of Ask1 through the Akt1-dependent phosphorylation of Ask1 P-Ser83. In these cells, Ask1 will turn on the activation of tolerable levels of JNK and p38, leading to a committed-to-regenerate status. Eventually, the JNK and p38 pathways will promote the transcription of the *unpaired* cytokines necessary for survival and regeneration [5,15,69–72]. In addition to cytokines, Wnt/Wg signaling pathway acts as a mitogenic signal involved in wing disc regeneration that responds to JNK [11,18,69,73] as well as the inhibition of the Hippo pathway, which results in increased Yki activity [74–76].

Several phosphatases are sensitive to ROS, and could be also integrated into the model presented here. This is the case of the JNK phosphatases, which are critical molecular targets of ROS. Oxidative inhibition of JNK phosphatases results in sustained JNK activation in order to promote caspase 3 cleavage and eventually cell death [77]. Therefore, the early burst of ROS in dying cells could activate lethal levels of JNK activity through inhibition of the *Drosophila* JNK phosphatase *puckered* concomitantly with the high levels of Ask1, and together boost the committed-to-die status. Additionally, the PTEN phosphatase, which regulates many cellular processes through direct antagonism of Pi3K signaling, is also ROS sensitive. Increasing ROS can enhance insulin signaling attributable to the oxidative inhibition of PTEN [78]. PTEN inactivation enhances the activation of AKT signaling, which in turn promotes the expression of cell survival genes [78]. Thus, PTEN could be involved in the living cells to release the Akt1 activity

that is necessary to reach the tolerable levels of Ask1/JNK/p38. Therefore, the low oxidative stress that reaches the living cells can trigger not only Trx2 dissociation from Ask1, but also the PTEN-oxidation dependent Pi3K/Akt1 activity required for attenuated Ask1 necessary for survival and proliferation.

Our model concerns to an inherent response in the imaginal disc epithelium. However, other signals could enhance or stabilize the ROS-dependent regenerative response. For example, ROS produced after wounding are necessary for recruitment and activation of the *Drosophila* macrophage-like immune cells, which will secrete the TNF ortholog Eiger. Eiger activates JNK signaling in the epithelial cells to contribute to the regenerative response [6].

Because the moderate activity of Ask1 for regeneration is achieved in an Akt1-dependent manner, and because Akt1 functions downstream of the insulin signaling, it seems conceivable an implication of nutrition in imaginal disc regeneration. The production and secretion of insulin and AKH, the fly analog of mammalian glucagon, vary depending of the food content. Thus, a challenge for the future will be to analyze how these hormones can modulate the regenerative response to damage. For example, the *Drosophila* TNF Eiger is produced by the fat body when larvae are exposed to low protein diet [79]. As Eiger activates JNK non-autonomously in many tissues, it could have a central role in imaginal disc regeneration, depending on food availability. The relation between nutrition and metabolism, as well as their involvement in regeneration deserves further analysis in order to achieve an integrative view of regeneration.

Materials and methods

Drosophila strains

The *Drosophila melanogaster* strains, *sal^{E/Pv}-LHG* and *lexO-rpr*, are previously described [5]; *lexO-GFP* (gift from K. Basler). *UAS-H2B-RFP* (from J. Knoblich), *Akt1¹* (gift from H. Stocker), *sal-Gal4*, *sal^{E/Pv}-Gal4* (gift from J.F. de Celis), *ci-Gal4* (from R. Holmgren) and *en-Gal4* (gift from G. Morata). The following strains were provided by the Bloomington Drosophila Stock Center: *tubGal80^{TS}* (RRID:BDSC_7017), *ptc-Gal4* (RRID:BDSC_2017), *Ask1^{MB06487}* (RRID:BDSC_26048) *Ask1^{MI02915}* (RRID:BDSC_36163), *UAS-GFP* (RRID:BDSC_4776), *UAS-dp110^{DN}* (RRID:BDSC_25918), *Df(3R)BSC636* (RRID:BDSC_25726), *UAS-Ask1^{RNAi}* (RRID:BDSC_35331), *UAS-Cat.A* (RRID:BDSC_24621), *UAS-Sod.A* (*Sod1*) (RRID:BDSC_24754), *UAS-rpr* on the X chromosome (RRID:BDSC_5823), *UAS-rpr* on the third chromosome (RRID:BDSC_50791). These strains are described in FlyBase: *hh-Gal4*, *ap-Gal4*, *UAS-hid*. The *UAS-Akt^{RNAi}* (2902) strain was obtained from the Vienna Drosophila Resource Center (VDRC). We used the *UAS-myr-Akt1.S* [50]. Canton S and *w¹¹⁸* were used as controls. A full list of genotypes is provided at the end of this section.

Genetic ablation and dual Gal4/LexA transactivation system

Cell death was genetically induced as previously described [5]. We used the *sal^{E/Pv}-Gal4* as a driver, which consists of the *spalt* wing enhancer with expression confined to the wing to score adult wing parameters. The UAS lines used to promote cell death were *UAS-rpr* or *UAS-hid*, two pro-apoptotic genes, controlled by the thermo-sensitive Gal4 repressor *tubGal80^{TS}*. We also used the *sal^{E/Pv}-LHG* and *LexO-rpr* strains [5] for genetic ablation, utilizing the same design as for Gal4/UAS.

Embryos were kept at 17°C until the 8th day/192 hours after egg laying to prevent *rpr* expression. They were subsequently moved to 29°C for 11 hours and then back to 17°C to allow tissue to regenerate. Two types of controls were always treated in parallel; individuals

without *rpr* expression (*UAS-GFP*, moved to 29°C for 11 hours) and individuals kept continuously at 17°C to avoid any transgene activation.

In most of the experiments shown here, we induced cell death for 11h. However the induction of cell death for testing the effects of signals emerging from the dying zone was longer. This is the case of the P-Akt experiment in Fig 3B and 3C and for the genetic scavenging of ROS (*Sod1:Cat*) (Fig 4G and S6 Fig), in which *sal>rpr* was activated for 24h.

In dual transactivation experiments, we used *sal^{E/Pv}-LHG LexO-rpr* to ablate the *sal^{E/Pv}* domain (abridged as *sal^{E/Pv}>rpr*), whereas Gal4 was used to express different transgenes under the control of Gal4 drivers (*ci-Gal4* for anterior compartment; *ap-Gal4* for dorsal compartment and *hh-Gal4* for posterior compartment).

Test for regenerated adult wings and statistics

To test the capacity to regenerate in different genetic backgrounds, we used adult wings emerged 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 hours. 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 with a complete set of veins and interveins, as markers of normal patterning. For each sample, we scored the percentage of individuals belonging to the “regenerated wings” class. We calculated the standard error of the sample proportion based on a binomial distribution (regenerated complete wing or not) $SE = \sqrt{p(1-p)/n}$, where *p* is the proportion of successes in the population.

Computational characterization of the fly Ser83 domain

Serine/threonine putative phosphorylation sites were determined by scanning the orthologous human/fly sequences with the *RxxRxx[ST]* domain motif for the Akt kinase (purple block on S2 Fig) and the degenerated pattern *xxRxx[ST]* (green blocks on S2 Fig). Strikingly the human Ser83 site was not found on *Drosophila melanogaster*; which was confirmed by using GeneWise (version wise2.4.1) [80] to map the M3K5_HUMAN protein sequence (Q99683), downloaded from UniProt [81], over a window of 10kbp centered at the Ask1 transcription start site; this sequence segment was retrieved from FlyBase FB2017_06 genome version ([82], chr3R:19,875,000–19,885,000[+]). The GeneWise output is provided as S1 Appendix, the software tool was not able to find a match on the fly genome for the initial 126 residues from the human amino term. In order to assess the relevance of the fly Ser83 two homology-based approaches were implemented. First, a search on PFAM database [83] returned the domain of unknown function DUF4071 (PF13281). The first amino acid positions for this domain are highly conserved across a set of diverse homologs, including fly Ask1-PC and human MAP3K5 but also paralogs sharing the domain. The consensus sequence for DUF4071 has been included on the right alignment block from S2 Fig for illustrative purpose. The second approach was considering a precomputed set of homolog sequences downloaded from EggNOG database (version 4.5) [84]. A cluster of homologous sequences already included fly Ask1 (KOG4279), providing a total of 338 protein sequences for 164 species; a total of 49 sequences were selected from that set, fly Ask1-PC isoform among them, that were also reliably annotated as orthologs of human MAP3K5. Fly Ask1-PB isoform protein sequence was retrieved from FlyBase and appended to the selected MAP3K5 orthologs. Then, the 50 protein sequences were aligned by MAFFT (version 7.271) [85] using the following

parameters: `maxiterate = 1000`, `localpair`, `op = 10`. The complete alignment is available from [S2 Appendix](#), from which two conserved blocks centered on the human and fly Ser83 domains were trimmed and processed with `TeXshade` [86] to produce the [S2 Fig](#). Note that on [S2 Fig](#) the domain highlighted in cyan, which includes the fly equivalent column highlighted in red, is highly conserved in a wide range of taxa, from sponges to humans. Other domains are conserved only within chordata or even tetrapoda species. Such conservation signature makes *Drosophila* Ser83 a good candidate for functional and mutational studies.

Generation of *UAS-Ask1^{WT}* and *UAS-Ask1^{S83A}* flies

pUAS-attb-Ask1^{WT} was constructed by cutting Ask1 cDNA EcoRI /BamHI from DGRC clone FI02066 and cloning it into pUAS-attb.

pUAS-attb-Ask1^{S83A}. Two serines in the 75 and 83 residues in the DUF4071 domain showed putative non-canonical AKT phosphorylation sites (ILTQQRPLSYHYGVRESF). Both residues are spatially exposed similarly to human Ser83 of ASK1, which makes them accessible to kinases. pUAS-attb-Ask1 S83A was constructed by mutating serine 83 to alanine by PCR using oligos Ask1Mut-Fwd and Ask1S83A-Rev for partial PCR1 and Ask1S83A-Fwd and Ask1Mut-Rev for partial PCR2. Complete PCR was performed using the two partial PCRs as templates with oligos Ask1Mut-Fwd and Ask1Mut-Rev. The complete PCR was then cut with EcoRI /PflMI and cloned into FI02066-cut EcoRI/PflMI. The mutation introduced a new StuI site that was used to check the mutated clones. Mutated Ask1S83A cDNA was then cut from EcoRI/BamHI and cloned into pUAS-attb. Both clones were injected by standard procedures in line zh-86Fb-attP and transgenic lines were selected.

Ask1Mut-Fwd: AAT ACA AGA AGA GAA CTC TGA ATA CGG AAT

Ask1Mut-rev: CGG CGG TGT GGT TTT GTG CAC AAA CCG ATC

Ask1S83A-Fwd: CGT TAG GGA GGC CTT CGG GAT GAA GGA GA

Ask1S83A-Rev: CGG CGG TGT GGT TTT GTG CAC AAA CCG ATC

Immunocytochemistry

Immunostaining was performed using standard protocols. The primary antibodies used in this study were the polyclonal Ask1 P-Ser83 (Santa Cruz Biotechnology sc-101633 1:100, which recognizes the conserved region surrounding human P-Ser83) and Ask1 P-Thr (Santa Cruz Biotechnology sc-109911 1:100, which labels the preserved region nearby mouse P-Thr845). We selected the anti-Ask1 P-Ser83 because of the following in vivo analysis: (1) its localization dropped under a *UAS-Ask1^{RNAi}* ([S3C Fig](#)); (2) Its localization increased after ectopic activation of Ask1^{WT} ([Fig 2G](#)); (3) Mutation in the *Drosophila* Ser83 residue (*UAS-Ask1^{S83A}*), inhibited its localization ([Fig 2H](#)); (4) Akt activation or repression, resulted in increased or decreased localization of P-Ser83 ([Fig 3E–3H](#)). Other antibodies used were: Ptc (DSHB, 1:100), P-p38 (Cell Signalling 1:50), Mmp1 (cocktail of three antibodies: DSHB 3A6B4, 5H7B11, 3B8D12 1:100), P-Akt (S473 Cell Signalling 1:100) and P-Histone-H3 (Millipore, 1:1000).

The anti-P-JNK used was the Anti-ACTIVE® JNK pAb, Rabbit, (V7931, Promega, 1:100). The images were taken using the Thermal LUT from the 3D Surface Plot of FIJI, with maximum values of 50% and minimum 24% to minimize the background. Calculation of pixel intensities was obtained from raw images.

Fluorescently labeled secondary antibodies were from ThermoFisher Scientific. Discs were mounted in SlowFade or ProLong (ThermoFisher Scientific) supplemented with 1 μM TO-PRO-3 (TP-3) or YO-PRO-1 (YP1) (Life Technologies) to label nuclei. For apoptotic cell detection, we used the TUNEL assay. We employed the fluorescently labeled Alexa Fluor®

647-aha-dUTP (ThermoFisher Scientific), incorporated using terminal deoxynucleotidyl transferase (Roche).

The number of mitotic cells per mm² (Fig 1C) was calculated after counting the number of P-Histone-H3 (P-H3) positive cells in the anterior compartment of the wing pouch and hinge, excluding the notum, for all the genotypes shown. Note that two genotypes in Fig 1C show the anterior compartment labeled with the transgene *ci-Gal4 UAS-GFP* (*ci>GFP*), whereas in the two *ci>Ask1^{RNAi}* experiments the anterior compartment was labeled with anti-Ci. In these experiments shown in Fig 1C, the induction of cell death or activation of transgenes was done for 16h at 29°C. The number of mitosis after analyzing the stacks of confocal images was calculated using Fiji software.

Imaginal disc culture and physical injury

Wing discs were dissected from third instar larvae in Schneider's insect medium (Sigma-Aldrich), and a small fragment was removed with tungsten needles. To visualize Mmp1 staining, the discs were cultured in Schneider's insect medium supplemented with 2% heat-activated fetal calf serum, 2.5% fly extract and 5 µg/ml insulin, for 5 hours at 25°C. For P-p38, discs were injured in Schneider's insect medium and immediately fixed and stained. *Ex vivo* images were taken using a Leica SPE confocal microscope and processed with Fiji software.

Ex vivo discs were also monitored live after a cut, using *puc-Gal4 UAS-GFP* as a JNK reporter, using the same medium. For nuclei visualization in *ex vivo* culture, we used NucRed Live647 (Life Technologies; 1 drop per slide) added 20 minutes before imaging.

ROS scavenging

To prevent ROS production in *sal^{E/Pv}>rpr* discs we pursued two different protocols. First, we inhibited ROS chemically (Fig 4A–4F). To do this, standard fly food was supplemented with the antioxidant N-acetyl cysteine (NAC 100 µg/ml; Sigma-Aldrich). NAC treatment was dispensed on the 7th day of development at 17°C. On the 8th day, experimental larvae were moved to 29°C for 11 hours to promote cell death, whereas controls were transferred to a vial with standard food and moved to 29°C for the same time period. Afterward, the larvae were move back to 17°C to allow tissue recovery. Second, we decreased ROS production genetically by the ectopic expression of the Sod1 and Catalase enzymes using a recombinant fly *UAS--Sod1:UAS-Cat* (*Sod1:Cat*) in the *sal^{E/Pv}>rpr* domain (Fig 4G and S6 Fig). For those experiments, *Sod1:Cat* and *rpr* were activated for 24 hours at 29°C.

Oxidative stress induction

Third instar larvae were transferred to vials with 5mL of special medium containing 1.3% UltraPureTM LMP agarose (Invitrogen), 5% sucrose (Fluka) and the desired concentration of 0.1% H₂O₂ (Merck) or 1ng/µl tunicamycin (Sigma-Aldrich). To avoid loss of oxidative capacity, these substances were added to the media at a temperature below 45°C. The larvae were fed for 2 hours prior to dissection and fixation of the discs. Controls without H₂O₂ or tunicamycin were always handled in parallel.

Quantitative real-time PCR analysis

Control (*hh>RFP*) and experimental (*hh>Ask1^{RNAi}*) conditions were induced for 16h at 29°C (192h after egg laying) using the *Gal4/tub-Gal80^{TS}* system. Wing imaginal discs (n = 40) were dissected after induction in three biological replicates for each condition. RNA was extracted with the Zymo Research ZR RNA MicroPerp (R1060/R1061) and RNA Clean and

Concentrator (R1015/R1016) kits following standard protocols. RT-PCR was performed using SYBR green Master Mix (Roche). Specific primers for *Ask1* and *Rps18* are detailed in [S1B Fig](#). $\Delta\Delta Ct$ method was used to normalize the data. *Ask1*^{RNAi} and control samples were normalized against the housekeeping gene *Rps18*. Average standard error of the mean (SEM) of the three biological replicates was computed for each one based on three technical replicates by $\Delta\Delta Ct$ method.

Genotypes

Fig 1.

- (A) $+/+ \rightarrow wUAS-rpr/+; sal^{E/Pv}-Gal4/+; tubGal80^{TS}/+$
 $Ask1^{M102915}/+ \rightarrow wUAS-rpr/+; sal^{E/Pv}-Gal4/+; tubGal80^{TS}/Ask1^{M102915}$
 $Ask1^{MB06487}/+ \rightarrow wUAS-rpr/+; sal^{E/Pv}-Gal4/+; tubGal80^{TS}/Ask1^{MB06487}$
 (B) $sal^{E/Pv}>rpr \rightarrow w; ci-Gal4/LexO-rpr; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-GFP$
 $ci>Ask1^{RNAi} \rightarrow w; ci-Gal4/LexO-GFP; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-Ask1^{RNAi}$
 $sal^{E/Pv}>rpr ci>Ask1^{RNAi} \rightarrow w; ci-Gal4/LexO-rpr; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-Ask1^{RNAi}$
 $ap>Ask1^{RNAi} \rightarrow w; ap-Gal4/LexO-GFP; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-Ask1^{RNAi}$
 $sal^{E/Pv}>rpr ap>Ask1^{RNAi} \rightarrow w; ap-Gal4/LexO-rpr; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-Ask1^{RNAi}$
 (C) $sal>GFP ci>RFP \rightarrow w; ci-Gal4/LexO-GFP; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-RFP$
 $sal>GFP ci>Ask1^{RNAi} \rightarrow w; ci-Gal4/LexO-GFP; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-Ask1^{RNAi}$
 $sal^{E/Pv}>rpr ci>GFP \rightarrow w; ci-Gal4/LexO-rpr; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-GFP$
 $sal^{E/Pv}>rpr ci>Ask1^{RNAi} \rightarrow w; ci-Gal4/LexO-rpr; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-Ask1^{RNAi}$

Fig 2.

- (B,C) WT $\rightarrow$ Canton S
 (D) $sal^{E/Pv}>rpr \rightarrow wUAS-rpr/+; sal^{E/Pv}-Gal4/+; tubGal80^{TS}/+$
 (E,F) $sal^{E/Pv}>rpr ci>Ask1^{RNAi} \rightarrow w; ci-Gal4/LexO-rpr; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-Ask1^{RNAi}$
 (G) $hh>Ask1^{WT}, GFP \rightarrow w; UAS-GFP/+; hh-Gal4/UAS-Ask1^{WT}$
 (H) $hh>Ask1^{S83A}, GFP \rightarrow w; UAS-GFP/+; hh-Gal4/UAS-Ask1^{S83A}$
 (I) $sal>GFP ci>Ask1^{WT} \rightarrow w; ci-Gal4/LexO-GFP; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-Ask1^{WT}$
 $sal>GFP ci>Ask1^{S83A} \rightarrow w; ci-Gal4/LexO-GFP; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-Ask1^{S83A}$
 $sal^{E/Pv}>rpr \rightarrow w; ci-Gal4/LexO-rpr; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-GFP$
 $sal^{E/Pv}>rpr ci>Ask1^{WT} \rightarrow w; ci-Gal4/LexO-rpr; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-Ask1^{WT}$
 $sal^{E/Pv}>rpr ci>Ask1^{S83A} \rightarrow w; ci-Gal4/LexO-rpr; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-Ask1^{S83A}$

Fig 3.

- A) Canton S
 (B) $ptc>rpr \rightarrow wUAS-rpr/+; ptc-Gal4: tubGal80^{TS}/+$
 (C) $sal>rpr \rightarrow wUAS-rpr/+; sal-Gal4/+; tubGal80^{TS}/+$
 (D,E) $ptc>Akt^{RNAi} \rightarrow w; ptc-Gal4:tubGal80^{TS}/+; UAS-Akt^{RNAi}/+$
 (F,G) $ptc>myrAkt \rightarrow w; ptc-Gal4:tubGal80^{TS}/+; UAS-myrAkt/+$
 (H-K) $sal^{E/Pv}>rpr hh>dp110^{DN} \rightarrow w; LexO-rpr/UAS-dp110^{DN}; sal^{E/Pv}-LHG:tubGal80^{TS}/hh-Gal4$
 (L) $ci>dp110^{DN} \rightarrow w; ci-Gal4/UAS-dp110^{DN}; sal^{E/Pv}-LHG:tubGal80^{TS}/+$
 $+/+ \rightarrow w; LexO-rpr/+; sal^{E/Pv}-LHG:tubGal80^{TS}/+$ (control for LexO-rpr in the second chromosome) and $wUASrpr/+; sal^{E/Pv}-Gal4; tubGal80^{TS}$ (control for mutant backgrounds)
 $sal^{E/Pv}>rpr ci>dp110^{DN} \rightarrow w; ci-Gal4/UAS-dp110^{DN}; sal^{E/Pv}-LHG:tubGal80^{TS}/LexO-rpr$
 $sal^{E/Pv}>rpr Akt^{1/+} \rightarrow wUAS-rpr/+; sal^{E/Pv}-Gal4; tubGal80^{TS}; Akt^{1/+}$
 $sal^{E/Pv}>rpr Ask1^{MB06487/+} Akt^{1/+} \rightarrow wUAS-rpr/+; sal^{E/Pv}-Gal4; tubGal80^{TS}; Akt^{1/+} Ask1^{MB06487}$

$sal^{E/Pv} > rpr \text{ Ask1}^{MI02915/+} \text{ Akt}^{1/+} \rightarrow wUAS-rpr/+; sal^{E/Pv}-Gal4; tubGal80^{TS}; Akt^1/Ask1^{MI02915}$

Fig 4.

(B,C) Std food and NAC $\rightarrow wUAS-rpr/+; sal^{E/Pv}-Gal4/+; tubGal80^{TS}/+$

(E,F) CTRL $\rightarrow w^{118}; +; +$

$Ask1^{-/-} \rightarrow w^{118}; +; Ask1^{MB06487}/Ask1^{MB06487}$

(G) $sal > rpr, GFP \rightarrow w; sal^{E/Pv}-Gal4/UAS-GFP; tubGal80^{TS}/UAS-rpr$

$sal > rpr, Sod1:Cat \rightarrow w; sal^{E/Pv}-Gal4/UAS-Sod1:UAS-Cat; tubGal80^{TS}/UAS-rpr$

Fig 5.

(A,C) $sal^{E/Pv} > rpr \rightarrow w; LexO-rpr/+; sal^{E/Pv}-LHG:tubGal80^{TS}/+$

(B,D) $sal^{E/Pv} > rpr \text{ ci} > Ask1^{RNAi} \rightarrow w; ci-Gal4/LexO-rpr; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-Ask1^{RNAi}$

(E-H) $en > Ask1^{RNAi}, GFP \rightarrow w; en-Gal4/UAS-GFP; UAS-Ask1^{RNAi}/+$

S1 Fig.

(A) $sal^{E/Pv} > rpr \text{ Ask1}^{MI02915/+} \rightarrow wUAS-rpr/+; sal^{E/Pv}-Gal4/+; tubGal80^{TS}/Ask1^{MI02915}$

$sal^{E/Pv} > rpr \text{ Ask1}^{MB06487/+} \rightarrow wUAS-rpr/+; sal^{E/Pv}-Gal4/+; tubGal80^{TS}/Ask1^{MB06487}$

$sal^{E/Pv} > rpr \text{ ci} > Ask1^{RNAi} \rightarrow w; ci-Gal4/LexO-rpr; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-Ask1^{RNAi}$

(B) Rt-q-PCR

Controls: $w; tubGal80^{ts}/+; hh-Gal4/UAS-RFP$

$UAS-Ask1^{RNAi}; w; tubGal80^{ts}/+; hh-Gal4/UAS-Ask1^{RNAi}$

S3 Fig.

(A) $w^{118}; +; +$

(B) $Ask1^+/Ask1^+ \rightarrow w^{118}; +; +$

$Ask1^{MB06487}/Ask1^{MB06487} \rightarrow w^{118}; +; Ask1^{MB06487}/Ask1^{MB06487}$

$Ask1^{MB06487}/Def(3R)BSC636 \rightarrow w^{118}; +; Ask1^{MB06487}/Def(3R)BSC636$

(C) $hh > Ask1^{RNAi}, GFP \rightarrow w; UAS-GFP/+; hh-Gal4/UAS-Ask1^{RNAi}$

S4 Fig.

(A) wt and $ptc > rpr \rightarrow wUAS-rpr/+; ptc-Gal4; tubGal80^{TS}/+$

(B) $sal^{E/Pv} > hid \rightarrow w; sal^{E/Pv}-Gal4/+; tubGal80^{TS}/UAS-hi$

(C) wt and physically injured $\rightarrow$ Canton S

S5 Fig.

(A-D) $sal^{E/Pv} > rpr \text{ ci} > dp110^{DN} \rightarrow w; ci-Gal4/UAS-dp110^{DN}; sal^{E/Pv}-LHG:tubGal80^{TS}/LexO-rpr$

S6 Fig.

$w; sal^{E/Pv}-Gal4/UAS-Sod1:UAS-Cat; tubGal80^{TS}/UAS-rpr$

S7 Fig.

(A) wt $\rightarrow$ Canton S

$sal^{E/Pv} > rpr \rightarrow w; LexO-rpr/+; sal^{E/Pv}-LHG:tubGal80^{TS}$

$sal^{E/Pv} > rpr, ci > Ask1^{RNAi} \rightarrow w; ci-Gal4/LexO-rpr; sal^{E/Pv}-LHG:tubGal80^{TS}/UAS-Ask1^{RNAi}$

(C) $w; UAS-GFP/+; puc-Gal4/+$

(D) $w; UAS-GFP/+; puc-Gal4/Ask1^{MB06487}$

Supporting information

S1 Fig. Supporting information for Fig 1. (A) Spectrum of wings with anomalous regeneration, ranging from complete regeneration ($sal^{E/Pv} > rpr$ in wild type, top) to a severe effect found after $Ask1^{RNAi}$ ($sal^{E/Pv} > rpr$ in mutant conditions, bottom). It includes a range of defects found after inducing cell death in Ask1 heterozygous background and after $UAS-Ask1^{RNAi}$. Arrowheads indicate some defects. (B) Real time quantitative PCR of the $UAS-Ask1^{RNAi}$. The table indicates the primers used. The RT-qPCR analysis is shown for Ask1 expression in wing

discs of controls (*hh>RFP*) and *Ask1^{RNAi}* (*hh>UAS-Ask1^{RNAi}*). qPCR results are presented as relative quantity of mRNA between control and *Ask1^{RNAi}*. Error bars represent the standard error of the mean from three biological replicates (n = 40 discs, each replicate).

(TIF)

S2 Fig. Alignment of Ask1 *D. melanogaster* orthologs focusing on human and fly Ser83 residues. Two blocks of conserved columns pinpointing the aforementioned residues were selected from the whole-length alignment of 50 MAP3K5/Ask1 protein orthologs provided on the [S2 Appendix](#). From top to bottom: sequence logos displaying information content based on amino acid frequencies; an estimate of hydrophobicity of the amino acid residues; the aligned sequences split into chordate and invertebrate sequences, above or below the two *Drosophila melanogaster* Ask1 isoforms respectively; finally, the similarity is depicted as a color gradient ranging from low (blue) to high (red). Bottom reference coordinates for the aligned residues are based on human reference sequence, which is listed on the first row. Serines, threonines, and arginines were labeled in red, green and blue when appearing outside the domain blocks, but shown in white and boldface within those blocks. Numbers on the left side of the alignment blocks correspond to the relative position of the first amino acid within those blocks for each species sequences. Left alignment block contains a domain in purple that matches the consensus RxxRxx [ST] pattern, although conservation is limited to birds and mammals (dark versus light shades of violet). On the right, the domain highlighted in cyan is conserved all across the diverse taxa, from human (top) to sponges (bottom), and it contains a serine (red background) that corresponds to the fly Ser83. On both panels, another domain matching a degenerated xxRxx [ST] pattern that is only conserved within vertebrate sequences is shown as a green block. Right alignment also includes at the bottom the consensus sequence of a PFAM domain of unknown function (DUF4071) that starts two residues before the Ser83 conserved block; this block within the domain shows high conservation across the different protein families considered when building the corresponding model.

(TIF)

S3 Fig. Supporting information for Fig 2. (A) Mitotic (P-H3 positive) and Ask1 P-Thr. Lower right images: Zoom of mitotic cells from the white square. TP-3: TO-PRO-3 nuclear staining. (B) The basal levels of P-Thr in wild-type (WT) discs (mitotic and interphase cells) decrease in the *Ask1^{MB06487}* homozygous mutant background and are strongly reduced in the *Ask1^{MB06487}* mutant background over the deficiency *Def(3R)50636*. Quantification of mean pixel intensity for P-Thr in mitotic and for P-Thr in non-mitotic (basal levels) cells are shown in separate graphics. P-Thr mean Pixel intensity in mitotic cells of the wild-type discs (+/+), 113.92±20.60, S.D. (n = 10); of the *Ask1^{MB06487}/Ask1^{MB06487}* discs, 104.43±9.54, S.D. (n = 10); and of the *Ask1^{MB06487}/Def(3R)BSC636* 88.37±13.03, S.D. (n = 10). Mean pixel intensity of the basal levels in interphase cells of the wild-type, 50.44±6.27, S.D. (n = 20); of the *Ask1^{MB06487}/Ask1^{MB06487}*, 41.65±4.80, S.D. (n = 10); and of the *Ask1^{MB06487}/Def(3R)BSC636*, 27.22±5.73, S.D. (n = 18). (C) Inhibition of the endogenous Ask1 P-Ser83 levels after expressing the *UAS-Ask1^{RNAi}* in the posterior compartment, using the *hh-Gal4* driver. Scale bars: 5µm.

(TIF)

S4 Fig. Supporting information for Fig 2. (A) Left: wild-type disc stained with P-Ser83. Right: disc in which apoptosis has been induced with the driver *ptc-Gal4 UAS-rpr* (*ptc>rpr*) and stained with P-Ser83 (n = 5). (B) *Sal^{E/Pv}>hid* discs stained with P-Thr (n = 12) and P-Ser83 (n = 5). YP-1: YO-PRO-1 nuclear staining. Purple: *sal^{E/Pv}>rpr* ablated zone. (C) Staining of Ask1 P-Ser83 after physical injury, (n = 6). Square:

magnified image. Scale bars: 50µm.
(TIF)

S5 Fig. Supporting information for Fig 3. (A) Design of the experiments in B, C and D with *sal^{E/Pv}>rpr* cell death (purple) and blocking Pi3K (*ci>dp110^{DN}*) in the anterior compartment (black). (B-D) Staining of P-Akt (B), Ask1 P-Ser83 (C) and Ask1 P-Thr (D). White-dotted line indicates anterior-posterior boundary. TP3: TO-PRO-3 nuclear staining. Scale bars: 50µm.
(TIF)

S6 Fig. TUNEL assay and P-Thr of discs with simultaneous *Sod1:Cat* and *rpr* activation (*sal^{E/Pv}>rpr, Sod1:Cat*; low oxidative stress). Purple area shows where transgenes were activated.
(TIF)

S7 Fig. Supporting information for Fig 5. (A) P-JNK staining in wild type, *sal^{E/Pv}>rpr*, and two examples of *sal^{E/Pv}>rpr ci>Ask1^{RNAi}*. Thermal LUT was used to detect pixel differences. TP-3: TO-PRO-3 nuclear staining. Purple indicates the dead zone. Colorized red indicates the *ci>Ask1^{RNAi}* zone. (B) Quantification of the P-JNK in raw images. A and P indicate the approximate areas measured for pixel intensity in n = 15 for each genotype. (C-E) Live imaging of a *puc-Gal4, UAS-GFP* used as a reporter of the JNK pathway. Discs were cut and cultured for 6 hours. White lines indicate the wound site. White arrows point to the endogenous *puc* expression zone at the tip of the notum. (C) Wild type. (D) *Ask1^{MB06487}* heterozygous. The nuclear marker NucRed Live647 was added in the culture medium. Scale bar: 50µm. (E) Quantification of *puc-Gal4, UAS-GFP* reporter at the wound site (n = 13 discs each genotype). Error bars indicate standard deviation. **P<0.01, ***P<0.001.
(TIF)

S1 Appendix. Computational verification that human Ser83 domain is missing on *Drosophila melanogaster* Ask1 gene loci. This file contains the plain text *genewise* alignment of the human M3K5_HUMAN protein sequence against the genomic segment of 5kbp upstream/downstream that is centered on the annotated fly Ask1 gene transcription start site. No alignment was reported for the first 126 residues of the human protein where the Ser83 RxxRxx [ST] domain has been described.
(PDF)

S2 Appendix. Complete alignment of the selected 50 MAP3K5/Ask1 protein orthologs that illustrates high conservation across the sequence. Many positions are preserved from sponges to humans, including serine/threonine residues, whilst other show a dual pattern of conservation with specific substitutions between chordate and invertebrate species. From the whole set of 338 sequences on 164 species that define the EggNOG (<http://eggnogdb.embl.de>) homologs cluster KOG4279, comprising human MAP3K5 and its counterpart in fly Ask1-PC, we filtered out 49 proteins that were confidently annotated as MAP3K5 orthologs on different taxa plus fly Ask1-PB isoform downloaded from FlyBase, which are listed here (names shown in parentheses are those used on the alignment sequence labels and refer to UniProt identifiers when possible): *Homo sapiens* ENSP00000351908 (M3K5_HUMAN), *Pan troglodytes* ENSPTRP00000043317 (K7CFQ9_PANTR), *Gorilla gorilla* ENSGGOP00000022651 (G3S3K1_GORGO), *Macaca mulatta* ENSMMUP00000021508 (F7G2W1_MACMU), *Pongo abelii* ENSPPYP00000019077 (H2PKF0_PONAB), *Otolemur garnettii* ENSOGAP00000000498 (H0WGN9_OTOGA), *Callithrix jacchus* ENSCJAP00000015659 (F7GZH9_CALJA), *Rattus norvegicus* ENSRNOP00000051338 (D3ZW27_RAT), *Mus musculus* ENSMUSP000000093485 (M3K5_MOUSE), *Cavia porcellus* ENSCPOP00000003829 (H0V2Q4_CAVPO), *Bos taurus*

ENSBTAP00000011949 (F1MXH6_BOVIN), *Pteropus vampyrus* ENSPVAP00000009127 (ens9127_PTEVA), *Mustela putorius furo* ENSMPUP00000014726 (G9K9I6_MUSPF), *Loxodonta africana* ENSLAFP00000000494 (G3UDL3_LOXAF), *Echinops telfairi* ENSETEP00000012543 (ens12543_ECHTE), *Canis lupus familiaris* ENSCAFP00000000362 (F1PA02_CANFA), *Ailuropoda melanoleuca* ENSAMEP00000010579 (D2H0V2_AILME), *Tursiops truncatus* ENSTTRP00000002388 (ens2388_TURTR), *Dasypus novemcinctus* ENSDNOP00000014966 (ens14966_DASNO), *Sarcophilus harrisii* ENSSHAP00000014955 (G3WHQ0_SARHA), *Gallus gallus* ENSGALP00000022482 (F1NYS9_CHICK), *Meleagris gallopavo* ENSMGAP00000013731 (G1NK21_MELGA), *Taeniopygia guttata* ENSTGUP00000011202 (H0ZKT1_TAEGU), *Pelodiscus sinensis* ENSPSIP00000018784 (K7GES3_PELSI), *Xenopus tropicalis* ENSXETP00000005271 (F7A415_XENT), *Gasterosteus aculeatus* ENSGACP00000009628 (G3NWA9_GASAC), *Oreochromis niloticus* ENSONIP00000007016 (I3JDS6_ORENI), *Danio rerio* ENSDARP00000046640 (E7F2L3_DANRE), *Takifugu rubripes* ENSTRUP00000002505 (H2RQT9_TAKRU), *Tetraodon nigroviridis* ENSTNIP00000022322 (Q4RFA2_TETNG), *Branchiostoma floridae* JGI219597 (C3XTE7_BRAFL), *Drosophila melanogaster* FBpp0089256 (Ask1-PB_DROME) and FBpp0289484 (Ask1-PC_DROME), *D. ananassae* FBpp0120816 (B3LXS9_DROAN), *Culex quinquefasciatus* CPIJ008487-PA (B0WN02_CULQU), *Anopheles gambiae* AGAP000747-PB (QEA6_ANOGA), *Aedes aegypti* AAEL008311-PA (Q16Z55_AEDAE), *Bombyx mori* BGIBMGA010545-TA (E9JEG8_BOMMO), *Danaus plexippus* EHJ64716 (G6DP69_DANPL), *Apis mellifera* GB14238-PA (H9KAX6_APIME), *Nasonia vitripennis* NV13729-PA (K7IXA9_NASVI), *Acyrtosiphon pisum* ACYPI009039-PA (J9K801_ACYPI), *Tribolium castaneum* TC000430-PA (TCOGS2_TRICA), *Daphnia pulex* DappuP328832 (P32883_DAPPU), *Caenorhabditis elegans* F59A6.1 (NSY-1_CAEEL), *C. japonica* CJA14063 (K7I1N3_CAEJA), *C. briggsae* CBG02299 (A8WUN1_CAEER), *Strongylocentrotus purpuratus* SPU_010872tr (H3I9Y9_STRPU), *Amphimedon queenslandica* PAC_15725247 (15725247_AMPQE), *Trichoplax adhaerens* TriadP10277 (B3RYN2_TRIAD).

(PDF)

S3 Appendix. Data for the graphics of Fig 4.
(DOCX)

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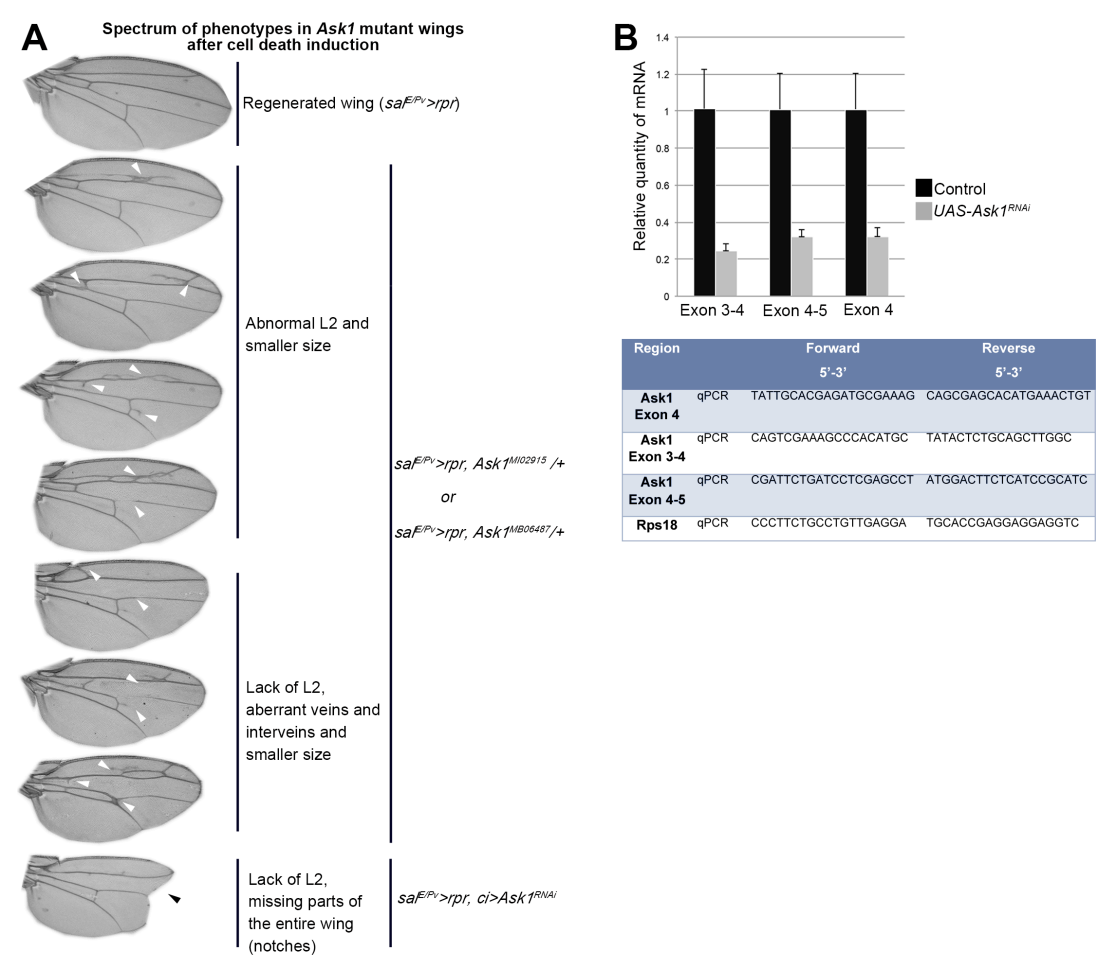
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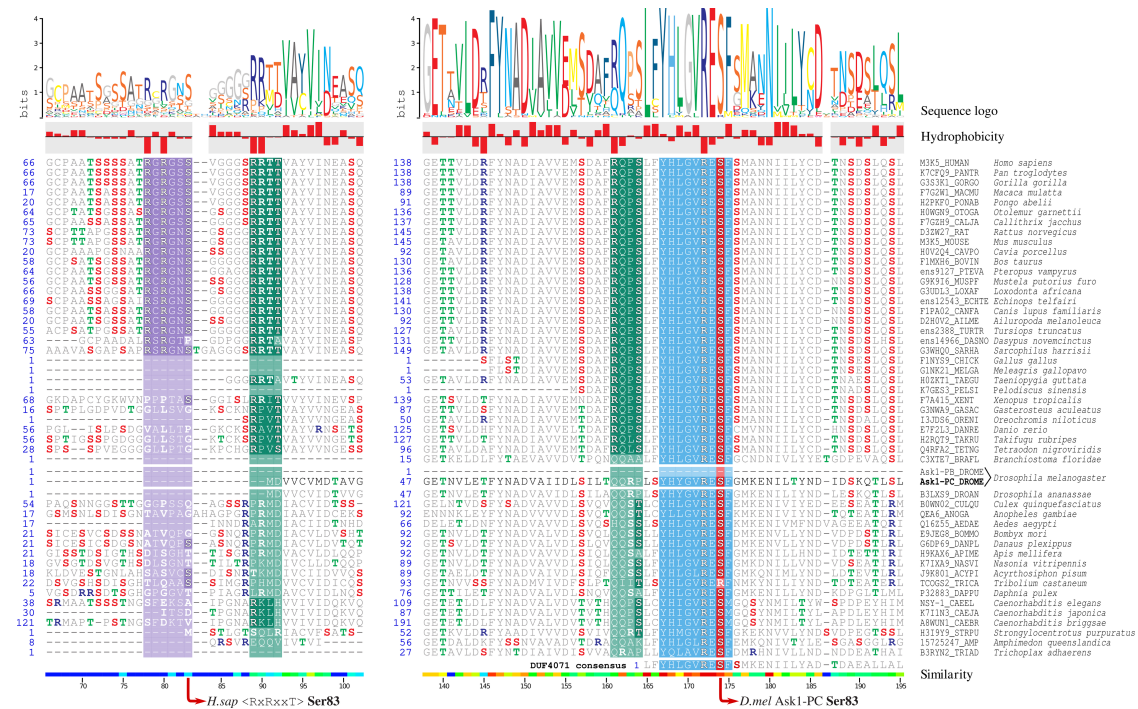
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S1 Fig. Supporting information for Fig 1.

(A) Spectrum of wings with anomalous regeneration, ranging from complete regeneration (*sal^{EPV}>rpr* in wild type, top) to a severe effect found after *Ask1^{RNAi}* (*sal^{EPV}>rpr* in mutant conditions, bottom). It includes a range of defects found after inducing cell death in *Ask1* heterozygous background and after *UAS-Ask1^{RNAi}*. Arrowheads indicate some defects. (B) Real time quantitative PCR of the *UAS-Ask1^{RNAi}*. The table indicates the primers used. The RT-qPCR analysis is shown for *Ask1* expression in wing discs of controls (*hh>RFP*) and *Ask1^{RNAi}* (*hh>UAS-Ask1^{RNAi}*). qPCR results are presented as relative quantity of mRNA between control and *Ask1^{RNAi}*. Error bars represent the standard error of the mean from three biological replicates (n = 40 discs, each replicate).

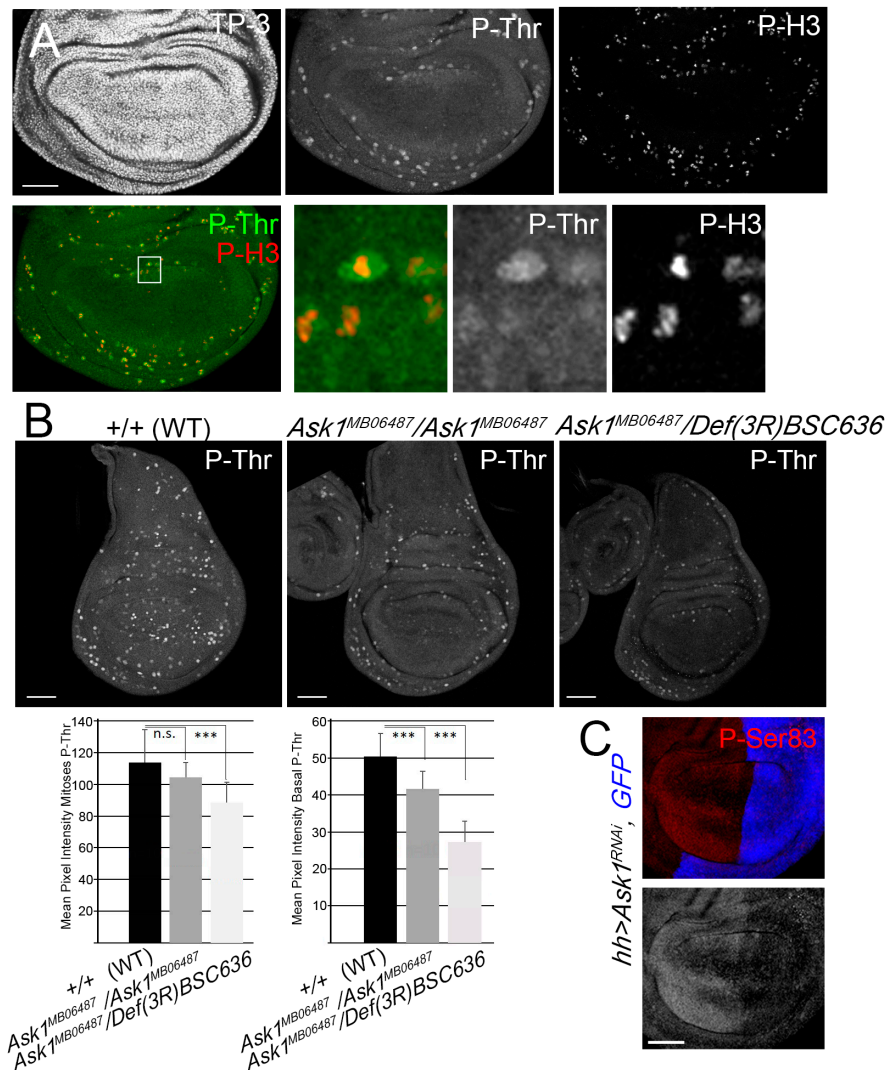
Supplementary material chapter 1



S2 Fig. Alignment of Ask1 *D. melanogaster* orthologs focusing on human and fly Ser83 residues.

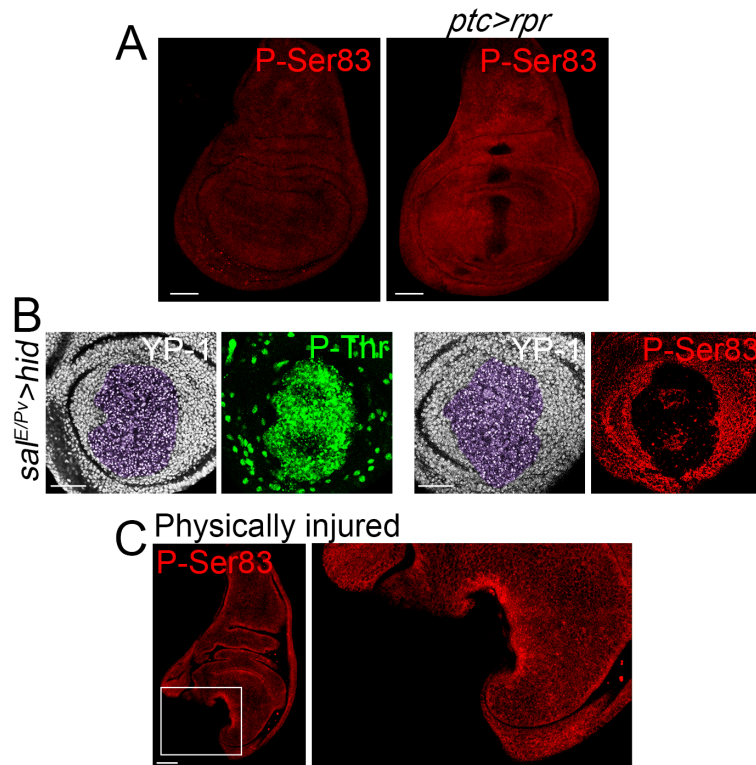
Alignment of Ask1 *D. melanogaster* orthologs focusing on human and fly Ser83 residues.

Two blocks of conserved columns pinpointing the aforementioned residues were selected from the whole-length alignment of 50 MAP3K5/Ask1 protein orthologs provided on the S2 Appendix. From top to bottom: sequence logos displaying information content based on amino acid frequencies; an estimate of hydrophobicity of the amino acid residues; the aligned sequences split into chordate and invertebrate sequences, above or below the two *Drosophila melanogaster* Ask1 isoforms respectively; finally, the similarity is depicted as a color gradient ranging from low (blue) to high (red). Bottom reference coordinates for the aligned residues are based on human reference sequence, which is listed on the first row. Serines, threonines, and arginines were labeled in red, green and blue when appearing outside the domain blocks, but shown in white and boldface within those blocks. Numbers on the left side of the alignment blocks correspond to the relative position of the first amino acid within those blocks for each species sequences. Left alignment block contains a domain in purple that matches the consensus RxRxx[ST] pattern, although conservation is limited to birds and mammals (dark versus light shades of violet). On the right, the domain highlighted in cyan is conserved all across the diverse taxa, from human (top) to sponges (bottom), and it contains a serine (red background) that corresponds to the fly Ser83. On both panels, another domain matching a degenerated xRxx[ST] pattern that is only conserved within vertebrate sequences is shown as a green block. Right alignment also includes at the bottom the consensus sequence of a PFAM domain of unknown function (DUF4071) that starts two residues before the Ser83 conserved block; this block within the domain shows high conservation across the different protein families considered when building the corresponding model.



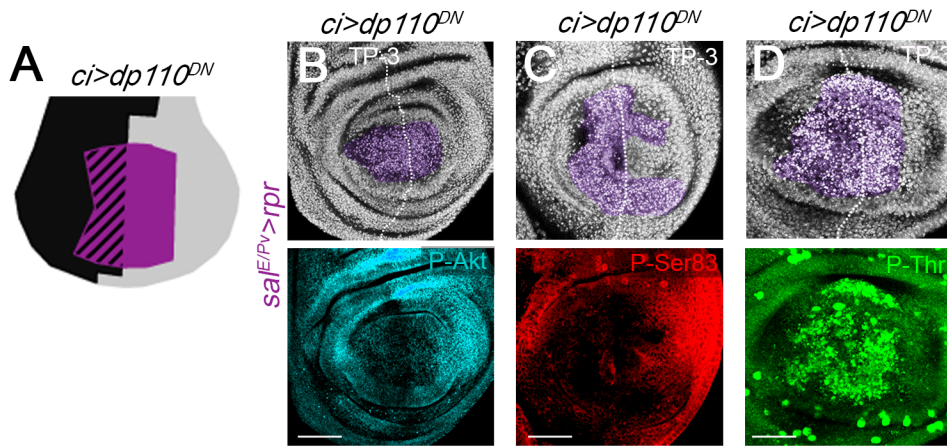
S3 Fig. Supporting information for Fig 2.

(A) Mitotic (P-H3 positive) and Ask1 P-Thr. Lower right images: Zoom of mitotic cells from the white square. TP-3: TO-PRO-3 nuclear staining. (B) The basal levels of P-Thr in wild-type (WT) discs (mitotic and interphase cells) decrease in the $Ask1^{MB06487}$ homozygous mutant background and are strongly reduced in the $Ask1^{MB06487}/Def(3R)BSC636$ mutant background over the deficiency $Def(3R)BSC636$. Quantification of mean pixel intensity for P-Thr in mitotic and for P-Thr in non-mitotic (basal levels) cells are shown in separate graphics. P-Thr mean Pixel intensity in mitotic cells of the wild-type discs ($+/+$), 113.92 ± 20.60 , S.D. ($n = 10$); of the $Ask1^{MB06487}/Ask1^{MB06487}$ discs, 104.43 ± 9.54 , S.D. ($n = 10$); and of the $Ask1^{MB06487}/Def(3R)BSC636$ 88.37 ± 13.03 , S.D. ($n = 10$). Mean pixel intensity of the basal levels in interphase cells of the wild-type, 50.44 ± 6.27 , S.D. ($n = 20$); of the $Ask1^{MB06487}/Ask1^{MB06487}$, 41.65 ± 4.80 , S.D. ($n = 10$); and of the $Ask1^{MB06487}/Def(3R)BSC636$, 27.22 ± 5.73 , S.D. ($n = 18$). (C) Inhibition of the endogenous Ask1 P-Ser83 levels after expressing the UAS-Ask1RNAi in the posterior compartment, using the hh-Gal4 driver. Scale bars: $5\mu m$.



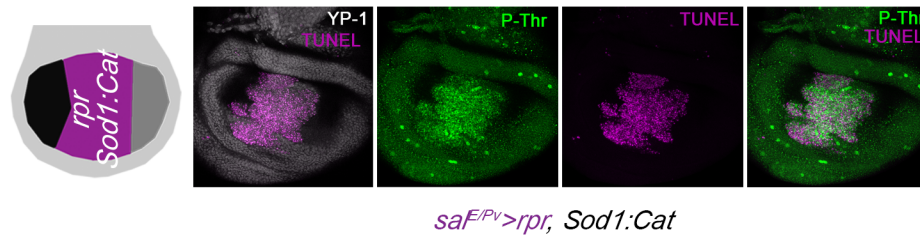
S4 Fig. Supporting information for Fig 2.

(A) Left: wild-type disc stained with P-Ser83. Right: disc in which apoptosis has been induced with the driver *ptc-Gal4 UAS-rpr* (*ptc>rpr*) and stained with P-Ser83 (n = 5). (B) *Sal^{E/Pv}>hid* discs stained with P-Thr (n = 12) and P-Ser83 (n = 5). YP-1: YO-PRO-1 nuclear staining. Purple: *sal^{E/Pv}>rpr* ablated zone. (C) Staining of Ask1 P-Ser83 after physical injury, (n = 6). Square: magnified image. Scale bars: 50μm.

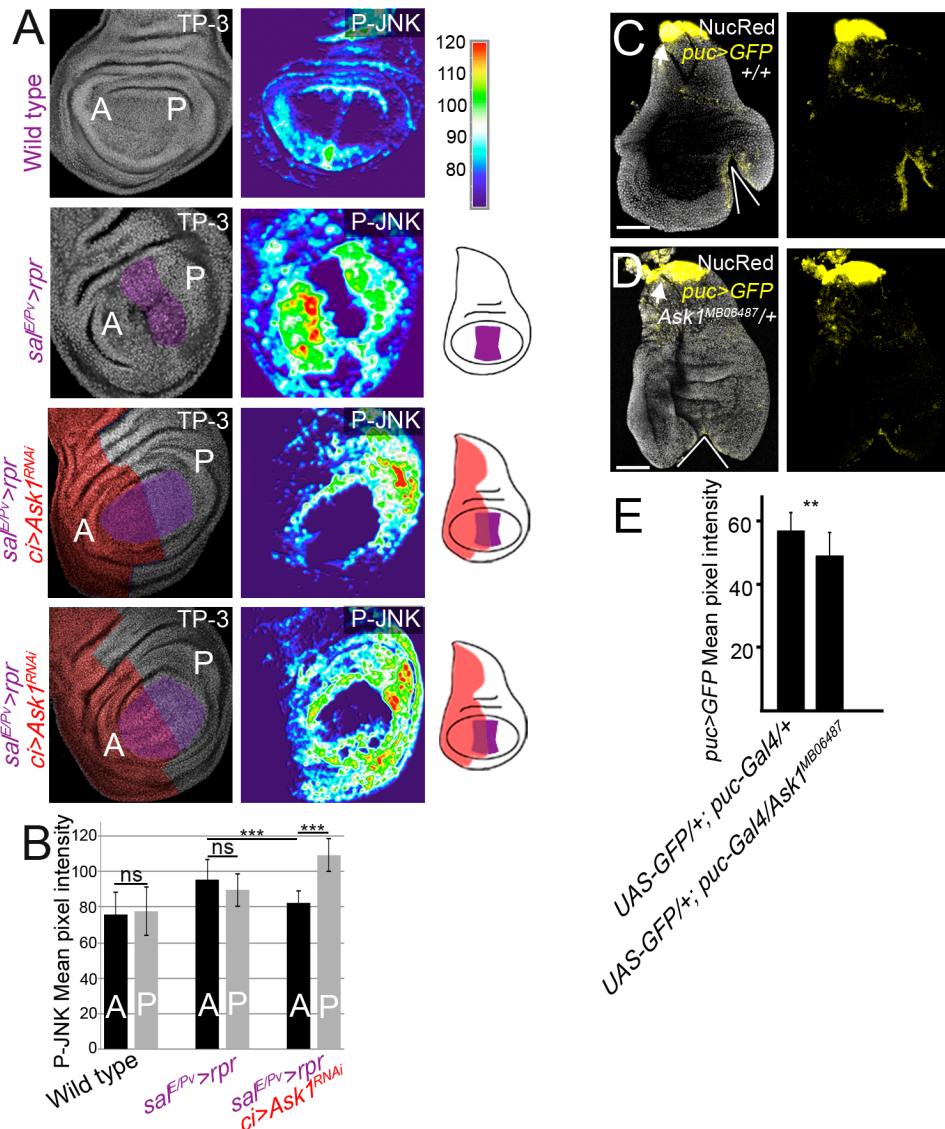


S5 Fig. Supporting information for Fig 3.

(A) Design of the experiments in B, C and D with *sal^{E/Pv}>rpr* cell death (purple) and blocking Pi3K (*ci>dp110^{DN}*) in the anterior compartment (black). (B-D) Staining of P-Akt (B), Ask1 P-Ser83 (C) and Ask1 P-Thr (D). White-dotted line indicates anterior-posterior boundary. TP3: TO-PRO-3 nuclear staining. Scale bars: 50μm.



S6 Fig. TUNEL assay and P-Thr of discs with simultaneous Sod1:Cat and rpr activation (*saE/Pv>rpr, Sod1:Cat*; low oxidative stress). Purple area shows where transgenes were activated.



S7 Fig. Supporting information for Fig 5.

(A) P-JNK staining in wild type, *salE/Pv>rpr*, and two examples of *salE/Pv>rpr ci>Ask1RNAi*. Thermal LUT was used to detect pixel differences. TP-3: TO-PRO-3 nuclear staining. Purple indicates the dead zone. Colorized red indicates the *ci>Ask1RNAi* zone. (B) Quantification of the P-JNK in raw images. A and P indicate the approximate areas measured for pixel intensity in $n = 15$ for each genotype. (C-E) Live imaging of a *puc-Gal4*, UAS-GFP used as a reporter of the JNK pathway. Discs were cut and cultured for 6 hours. White lines indicate the wound site. White arrows point to the endogenous *puc* expression zone at the tip of the notum. (C) Wild type. (D) *Ask1^{MB06487}* heterozygous. The nuclear marker NucRed Live647 was added in the culture medium. Scale bar: 50 μ m. (E) Quantification of *puc-Gal4*, UAS-GFP reporter at the wound site ($n = 13$ discs each genotype). Error bars indicate standard deviation. ** $P < 0.01$, *** $P < 0.001$.

S1 Appendix. Computational verification that human Ser83 domain is missing on *Drosophila melanogaster* Ask1 gene loci.

This file contains the plain text genewise alignment of the human M3K5_HUMAN protein sequence against the genomic segment of 5kbp upstream/downstream that is centered on the annotated fly Ask1 gene transcription start site. No alignment was reported for the first 126 residues of the human protein where the Ser83 RxRxx[ST] domain has been described.

Available at:

<https://doi.org/10.1371/journal.pgen.1007926.s008>

(PDF)

S2 Appendix. Complete alignment of the selected 50 MAP3K5/Ask1 protein orthologs that illustrates high conservation across the sequence.

Many positions are preserved from sponges to humans, including serine/threonine residues, whilst other show a dual pattern of conservation with specific substitutions between chordate and invertebrate species. From the whole set of 338 sequences on 164 species that define the EggNOG (<http://eggnogdb.embl.de>) homologs cluster KOG4279, comprising human MAP3K5 and its counterpart in fly Ask1-PC, we filtered out 49 proteins that were confidently annotated as MAP3K5 orthologs on different taxa plus fly Ask1-PB isoform downloaded from FlyBase, which are listed here (names shown in parentheses are those used on the alignment sequence labels and refer to UniProt identifiers when possible): Homo sapiens ENSP00000351908 (M3K5_HUMAN), Pan troglodytes ENSPTRP00000043317 (K7CFQ9_PANTR), Gorilla gorilla ENSGGOP00000022651 (G3S3K1_GORGO), Macaca mulatta ENSMUP00000021508 (F7G2W1_MACMU), Pongo abelii ENSPPYP00000019077 (H2PKF0_PONAB), Otolemur garnettii ENSOGAP00000000498 (H0WGN9_OTOGA), Callithrix jacchus ENSCJAP00000015659 (F7GZH9_CALJA), Rattus norvegicus ENSRNOP00000051338 (D3ZW27_RAT), Mus musculus ENSMUSP000000093485 (M3K5_MOUSE), Cavia porcellus ENSCPOP00000003829 (H0V2Q4_CAVPO), Bos taurus ENSBTAP00000011949 (F1MXH6_BOVIN), Pteropus vampyrus ENSPVAP00000009127 (ens9127_PTEVA), Mustela putorius furo ENSMPUP00000014726 (G9K9I6_MUSPF), Loxodonta africana ENSLAFP00000000494 (G3UDL3_LOXAF), Echinops telfairi ENSETEP00000012543 (ens12543_ECHTE), Canis lupus familiaris ENSCAF00000000362 (F1PA02_CANFA), Ailuropoda melanoleuca ENSAMEP00000010579 (D2H0V2_AILME), Tursiops truncatus ENSTTRP00000002388 (ens2388_TURTR), Dasypus novemcinctus ENSDNOP00000014966 (ens14966_DASNO), Sarcophilus harrisii ENSSHAP00000014955 (G3WHQ0_SARHA), Gallus gallus ENSGALP00000022482 (F1NYS9_CHICK), Meleagris gallopavo ENSMGAP00000013731 (G1NK21_MELGA), Taeniopygia guttata ENSTGUP00000011202 (H0ZKT1_TAEGU), Pelodiscus sinensis ENSPSIP00000018784 (K7GES3_PELSI), Xenopus tropicalis ENSXETP00000005271 (F7A415_XENT), Gasterosteus aculeatus ENSGACP00000009628 (G3NWA9_GASAC), Oreochromis niloticus ENSONIP00000007016 (I3JDS6_ORENI), Danio rerio ENSDARP00000046640 (E7F2L3_DANRE), Takifugu rubripes ENSTRUP00000002505 (H2RQT9_TAKRU), Tetraodon nigroviridis ENSTNIP00000022322 (Q4RFA2_TETNG), Branchiostoma floridae JGI219597 (C3XTE7_BRAFL), Drosophila melanogaster FBpp0089256 (Ask1-PB_DROME) and FBpp0289484 (Ask1-PC_DROME), D. ananassae FBpp0120816 (B3LXS9_DROAN), Culex quinquefasciatus CPIJ008487-PA (B0WN02_CULQU), Anopheles gambiae AGAP000747-PB (QEA6_ANOGA), Aedes aegypti AAEL008311-PA (Q16Z55_AEDAE), Bombyx mori BGIBMGA010545-TA (E9JEG8_BOMMO), Danaus plexippus EHJ64716 (G6DP69_DANPL), Apis mellifera GB14238-PA (H9KAX6_APIME), Nasonia vitripennis NV13729-PA (K7IXA9_NASVI), Acyrthosiphon pisum ACYPI009039-PA (J9K801_ACYPI), Tribolium castaneum TC000430-PA (TCOGS2_TRICA), Daphnia pulex DappuP328832 (P32883_DAPPU), Caenorhabditis elegans F59A6.1 (NSY-1_CAEEL), C. japonica CJA14063 (K7I1N3_CAEJA), C. briggsae CBG02299 (A8WUN1_CAEER), Strongylocentrotus purpuratus SPU_010872tr (H3I9Y9_STRPU), Amphimedon queenslandica PAC_15725247 (15725247_AMPQE), Trichoplax adhaerens TriadP10277 (B3RYN2_TRIAD).

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(PDF)

S3 Appendix. Data for the graphics of figure 4

Data for figure 4B Mean pixel intensities of P-Thr fluorescent labeling in the apoptotic zone of *sal^{E/Pv}>rpr* discs from larvae fed with a standard (dying cells 94.01±17.04; S.D., n=11) or NAC-supplemented (dying cells 31.07±9.38; S.D. n=10) diet.

Data for figure 4C. Mean pixel intensities of Ask1 P-Ser83 fluorescent labeling in the neighboring cells near the apoptotic zone of *sal^{E/Pv}>rpr* discs from larvae fed with a standard (living cells 57.56±23.27; S.D., n=20) or NAC-supplemented (living cells 29.54±5.92; S.D., n=18) diet.

Data for figure 4E. Left: P-Thr mean pixel intensity of wild-type wing discs from larvae fed with standard (79.36±18.65; S.D., n=17), H₂O₂- (93.37±3.87; S.D., n=7) and tunicamycin-supplemented (125.49±12.51; S.D., n=7) food. Right: P-Thr mean pixel intensity of *Ask1^{MB06487}* homozygous mutant discs from larvae fed with standard (92.92±9.75; S.D., n=6), H₂O₂- (83.66±7.11; S.D. n=11) and tunicamycin-supplemented (87.99±21.38; S.D., n=7) food.

Data for figure 4F. Left: P-Ser83 mean pixel intensity of wild-type wing discs from larvae fed with standard (79.14±17.69; S.D., n=19), H₂O₂- (110.89±15.43; S.D., n=14) and tunicamycin-supplemented (128.35±14.42; S.D., n=5) food.

Right: P-Ser83 mean pixel intensity of *Ask1^{MB06487}* homozygous mutant discs from larvae fed with standard (81.83±20.25; S.D., n=12), H₂O₂- (87.51±6.40; S.D., n=6) and tunicamycin-supplemented (90.27±13.26; S.D.; n=4) food.

Data for figure 4G. P-Akt mean pixel intensity for *sal^{E/Pv}>rpr*, *GFP* was 100.61±25.14, S.D. in A and 92.64±21.38, S.D. in P; and for *sal^{E/Pv}>rpr*, *Sod1:Cat* was 68.04±18.16, S.D. in A and 63.14±15.66, S.D. in P. P-Ser83 mean pixel intensity for *sal^{E/Pv}>rpr*, *GFP* was 86.89±34.49, S.D. in A and 78.46±31.06, S.D. in P; and for *sal^{E/Pv}>rpr*, *Sod1:Cat* was 63.25±23.82, S.D. in A and 54.32±19.92, S.D. in P. N= 23 discs for each genotype.

Chapter 2

*“...just as the sun strikes raindrops at a certain angle and throws a prism of color across the sky - so the space where I exist, and I want to keep existing, and to be quite frank I hope I die in, is exactly this middle distance: where **despair** struck pure **otherness** and created something **sublime**.”*

Donna Tartt, *The Goldfinch*, 2013

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

José Esteban-Collado, Montserrat Corominas and Florenci Serras*

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; Santabàrbara-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).

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; Santabàrbara-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; Santabàrbara-Ruiz et al., 2019). Phosphorylation of p38 is an early response to damage that occurs alongside JNK (Santabàrbara-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; Perdiguer 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 (Santabàrbara-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 (Santabàrbara-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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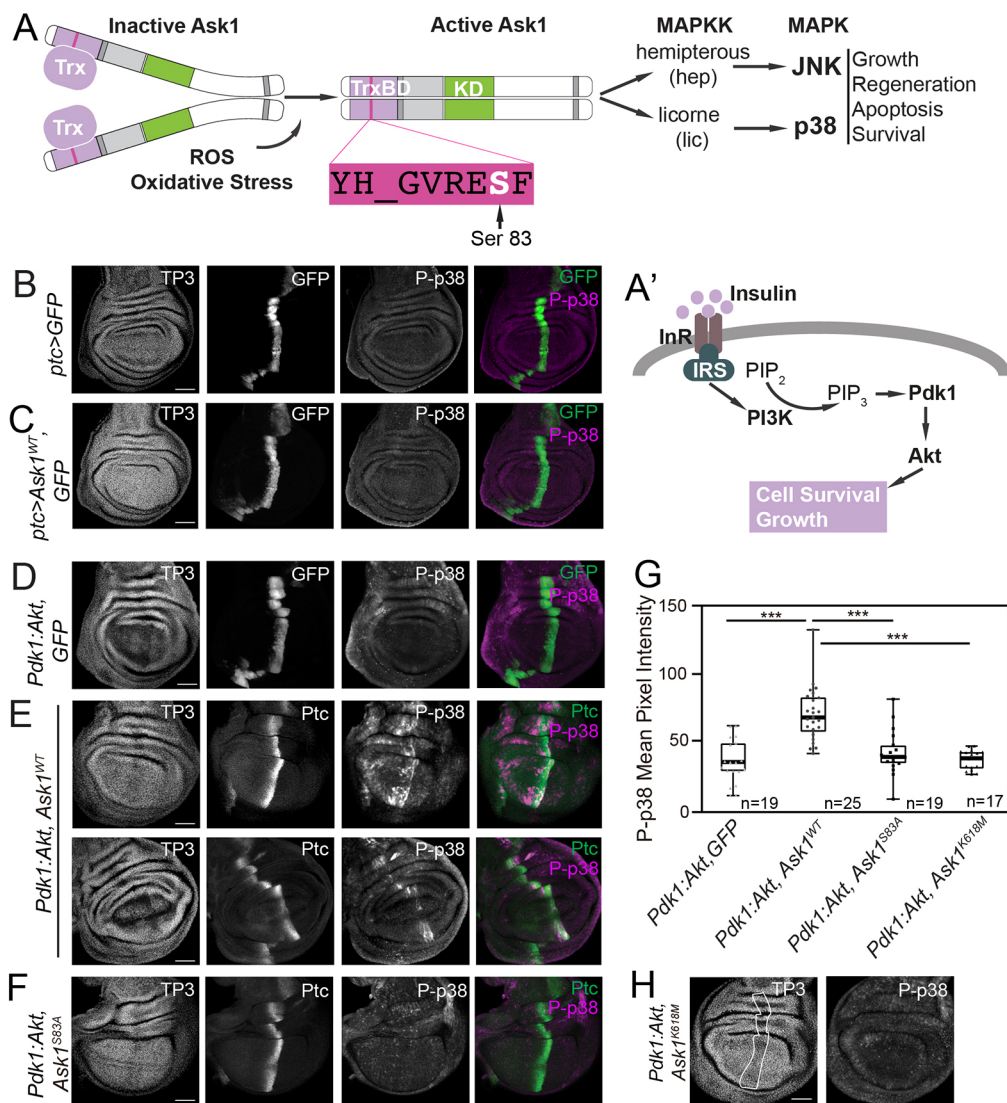


Fig. 1. The co-expression of Ask1 and Pdk1:Akt results in the activation of p38. (A) Ask/p38/JNK signaling. (A') Insulin/PI3K/Akt signaling. (B-D) P-p38 in (B) GFP-expressing control, (C) wild-type *Ask1^{WT}*, and (D) *Pdk1:Akt* and GFP. (E) Two examples of P-p38 after co-expressing *Pdk1:Akt* and *Ask1^{WT}*. (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 μ m.

(Santab rbara-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 *Ask1^{WT}*,

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 (Santabábara-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 phenotypic 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

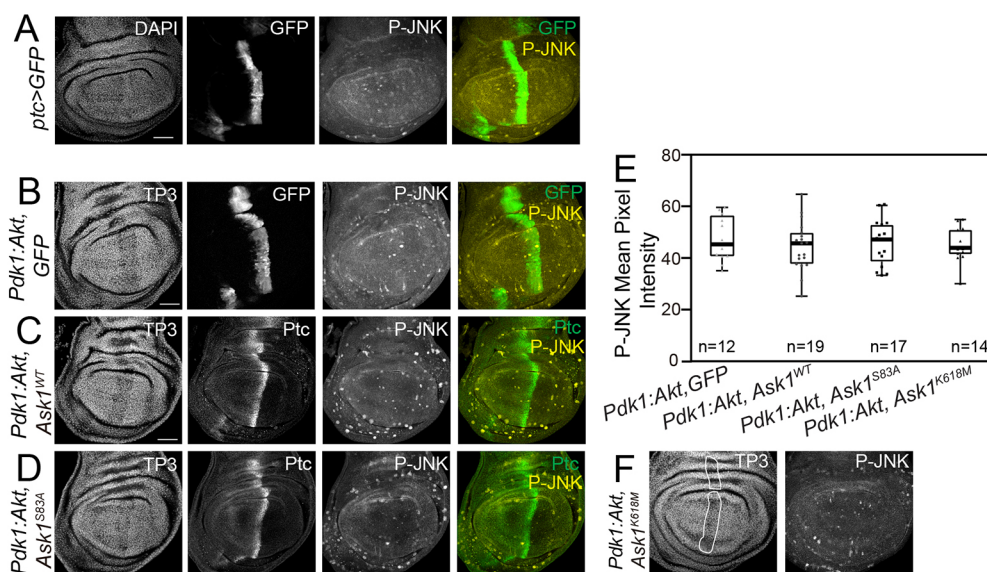


Fig. 2. The co-expression of *Ask1* and *Pdk1:Akt* does not result in the activation of JNK. (A) P-JNK in GFP-expressing control. (B-D) P-JNK after co-expression of (B) *Pdk1:Akt* and GFP, (C) *Pdk1:Akt* and *Ask1^{WT}*, or (D) *Pdk1:Akt* and *Ask1^{S83A}*. (E) Mean pixel intensity of P-JNK for the genotypes 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 μ m.

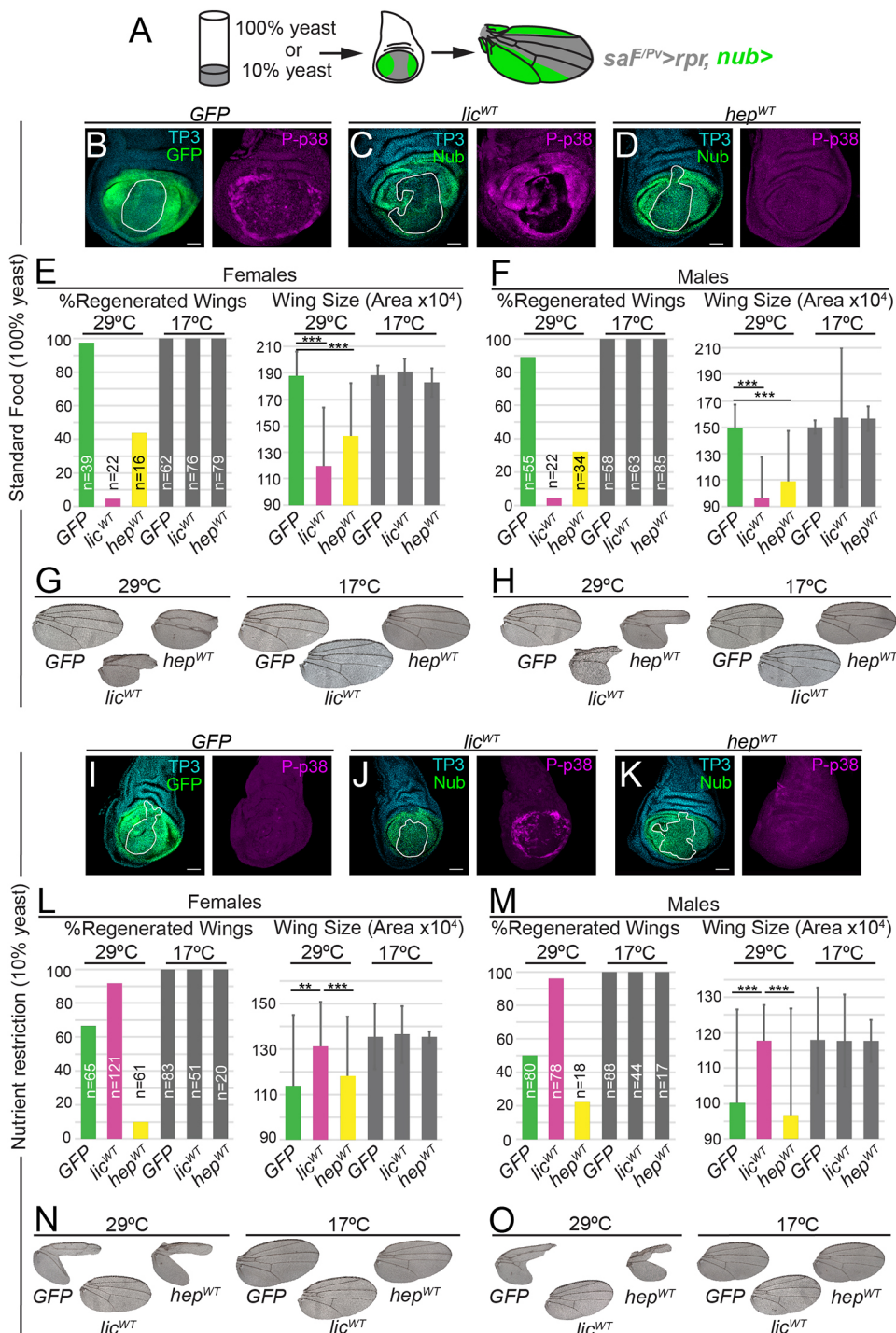


Fig. 3. The MAPKK *licorne* (*lic^{WT}*), but not *hemipterous* (*hep^{WT}*), reverses the failure in regeneration caused by nutrient restriction. (A) Zones of dual transactivation. Gray, apoptotic zone generated by *sa^{E/PV}>rpr*; green, transgene activation under *nub-Gal4* (*nub>*) to express the 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 μ m.

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

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 (Santabàrbara-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 (Santabàrbara-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^{EL/PV}-LHG*, *lexO-rpr*, *UAS-Ask1^{WT}* and *UAS-Ask1^{S83A}* *Drosophila melanogaster* strains have been previously described (Santabàrbara-Ruiz et al., 2015; Santabàrbara-Ruiz et al., 2019), as have *UAS-lic^{WT1.1}*

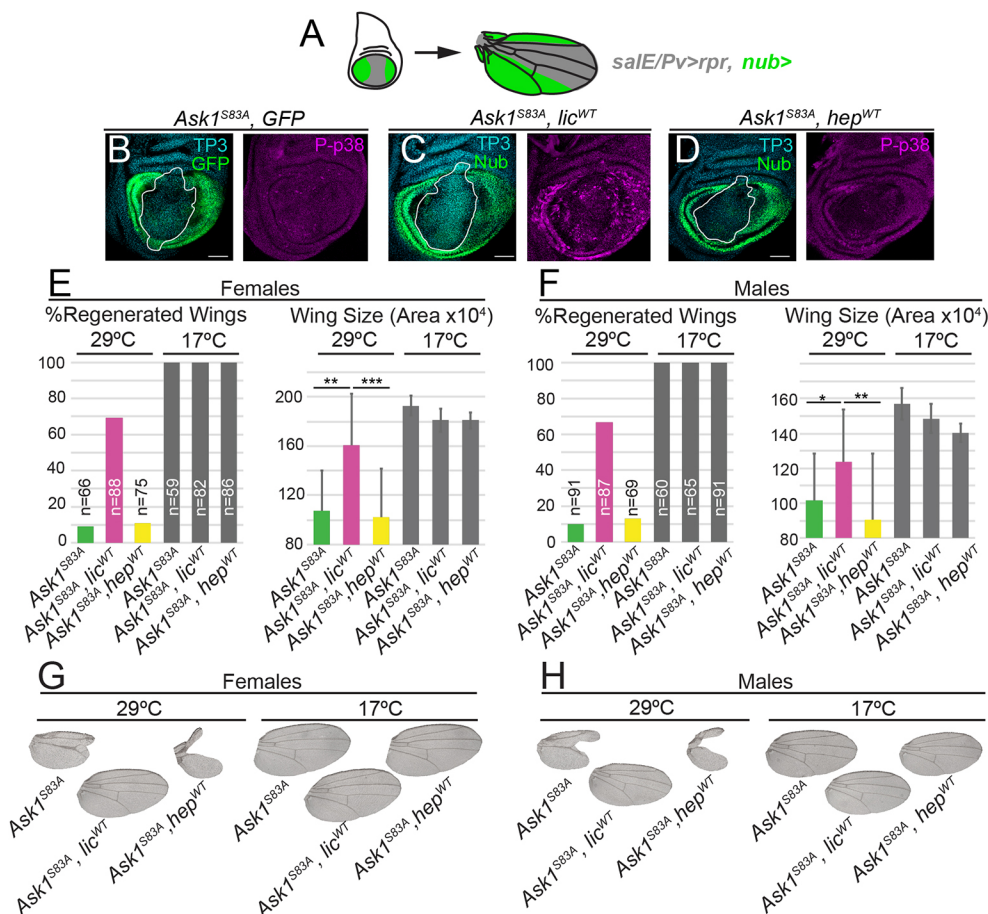


Fig. 4. The MAPKK *licorne* (*lic^{WT}*), but not *hemipterous* (*hep^{WT}*), reverses the failure in regeneration caused by *Ask^{S83A}* expression. (A) Zones of dual transactivation. Gray, apoptotic zone (*sal^{EL/PV}>rpr*); green, *nub>* zone of UAS-transgene expression. (B-D) P-p38 of *nub>Ask1^{S83A}, 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.

(Terriente-Félix et al., 2017), *UAS-hep^{WT}* (Uhlírova 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* (Santabàrbara-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: * $P<0.05$, ** $P<0.01$, *** $P<0.001$.

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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.

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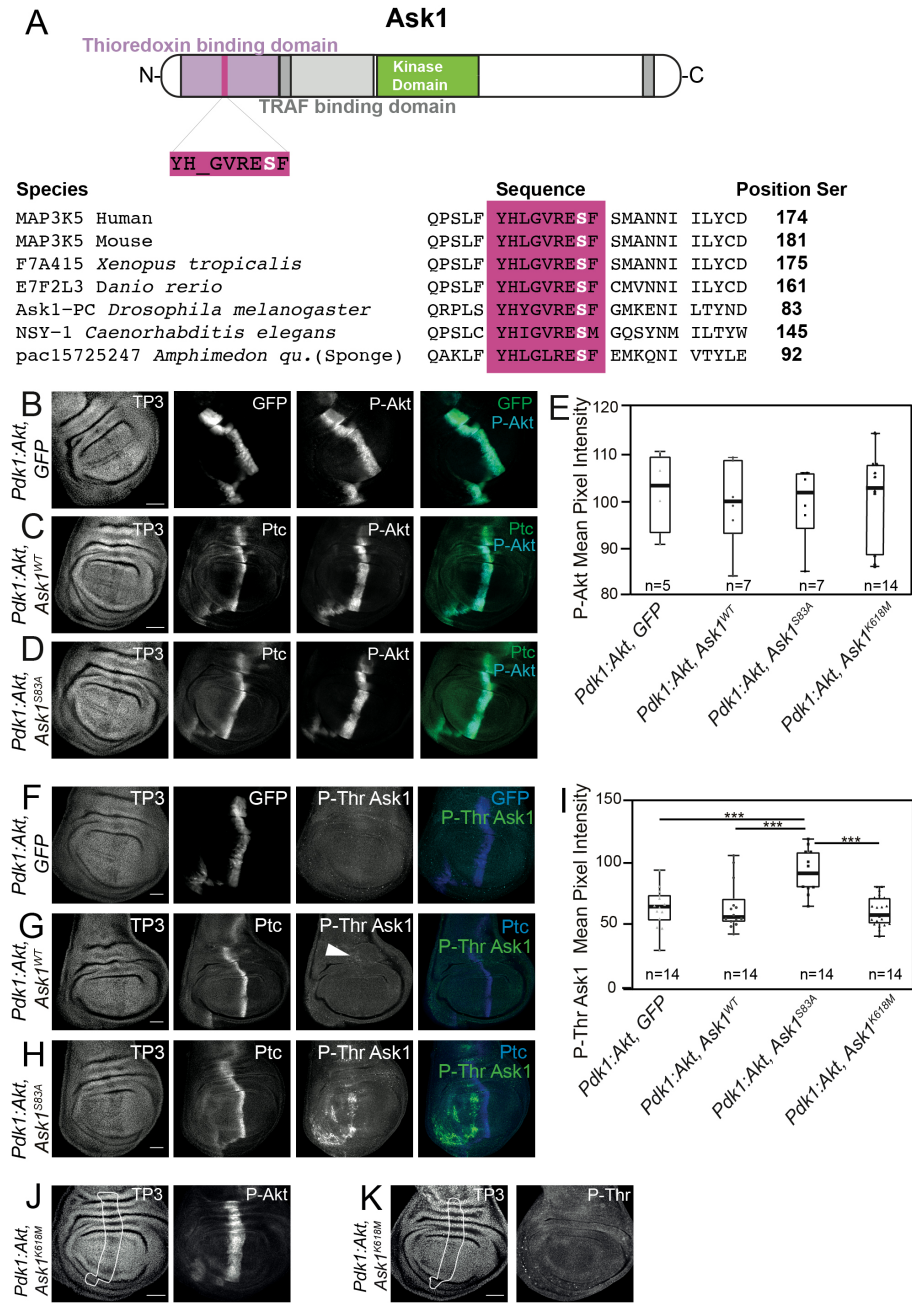


Figure S1. (A) The main domains of a canonical Ask1 protein. The position of the YH_GVRESF sequence is indicated, as well as the Ser83 residue of the *Drosophila* Ask1 protein. Conservation between some organisms is shown. Note the consensus YH_GVRESF sequence. Full comparative sequences of the Ask1 proteins are published in (Santabábara-Ruiz et al., 2019). Thioredoxin is responsive to oxidative stress and dissociates from the thioredoxin-domain to promote Ask1 activation. Dark gray, the C- and N-terminal coiled-coil domains involved in homodimerization. (B-E) Anti-P-Akt antibody staining to show the expression of *Pdk1:Akt* of the transgene

combinations indicated. (B) *Pdk1:Akt* and *GFP* co-expression; (C) *Pdk1:Akt* and *Ask1^{WT}* co-expression; and (D) *Pdk1:Akt* and *Ask1^{S83A}* co-expression. (E) The mean pixel intensity and quantification of the P-Akt immunofluorescence for the genotypes indicated. (F-H) Anti-P-Thr Ask1 antibody staining to show the expression of Ask1 of the transgene combinations indicated. (F) *Pdk1:Akt* and *GFP* co-expression; (G) *Pdk1:Akt* and *Ask1^{WT}* co-expression; and (H) *Pdk1:Akt* and *Ask1^{S83A}* co-expression. (I) The mean pixel intensity and quantification of the P-Thr Ask1 immunofluorescence for the genotypes indicated. The GFP staining corresponds to the controls. The others were stained with anti-Ptc antibody to identify the expression zone. Arrowhead points to a zone with some weak P-Thr Ask1 staining. (J) P-Akt immunofluorescence following the co-expression of *Pdk1:Akt* and *Ask1^{K618M}*. (K) P-Thr Ask1 immunofluorescence following the co-expression of *Pdk1:Akt* and *Ask1^{K618M}*. The stripe outlined in white indicates the zone of transgene expression. TP-3 (TO-PRO-3): nuclear staining. Scale bars: 50 μ m. *** $p < 0.001$.

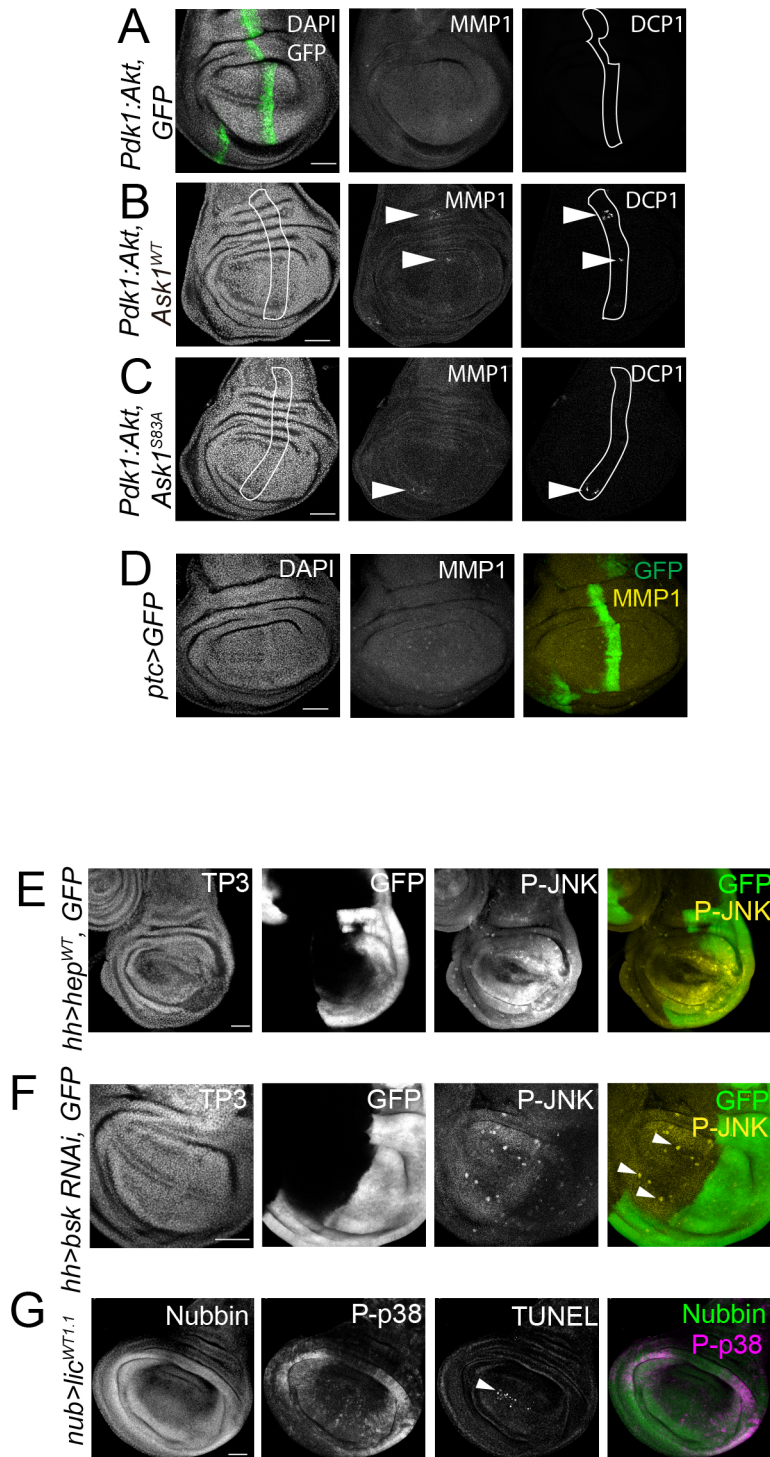


Figure S2. Apoptosis measured by assessing the expression of the caspase DCP1 and MMP1 after the co-expression of (A) *Pdk1:Akt* and *GFP*, (B) *Pdk1:Akt* and *Ask1^{WT}*, or (C) *Pdk1:Akt* and *Ask1^{S83A}*, using the *ptc>* driver (outline). The arrows indicate some apoptotic cells, which were also positive for MMP1. (D) Endogenous expression of MMP1 in *ptc>GFP* wing discs. (E-G) Validation of antibodies against proteins in the JNK and p38 pathways. (E) Anti-P-JNK antibody staining after the ectopic expression of the wild-type JNKK *hemipterous* (*hep^{WT}*). (F) Anti-P-JNK antibody staining after the administration of RNAi against the JNK *basket* (*bsk RNAi*). The Gal4 driver used in

these experiments was *hh-Gal4*, which controls expression in the posterior compartment, allowing us to compare the staining with that of the anterior compartment. Note that for *hep^{WT}*, the posterior compartment was smaller, likely due to apoptosis, with strong P-JNK staining. Following the expression of the *bsk RNAi*, endogenous P-JNK levels decreased. Moreover, P-JNK in mitoses (arrowheads) was reduced in the posterior (GFP) compartment. Note that both transgenes in (E) and (F) were co-expressed with *UAS-GFP*. (G) Expression of the wild-type MAPKK *licorne* (*lic^{WT}*), stained with the anti-P-p38 antibody. The driver used here was *nub-Gal4*, which operates in the entire wing pouch. The same driver was used for the rescue experiments in Figures 3 and 4. The arrowhead indicates dead cells. An antibody against Nubbin was used to identify the *nub-Gal4* domain. TP-3 (TO-PRO-3): nuclear staining. Scale bars: 50 μ m.

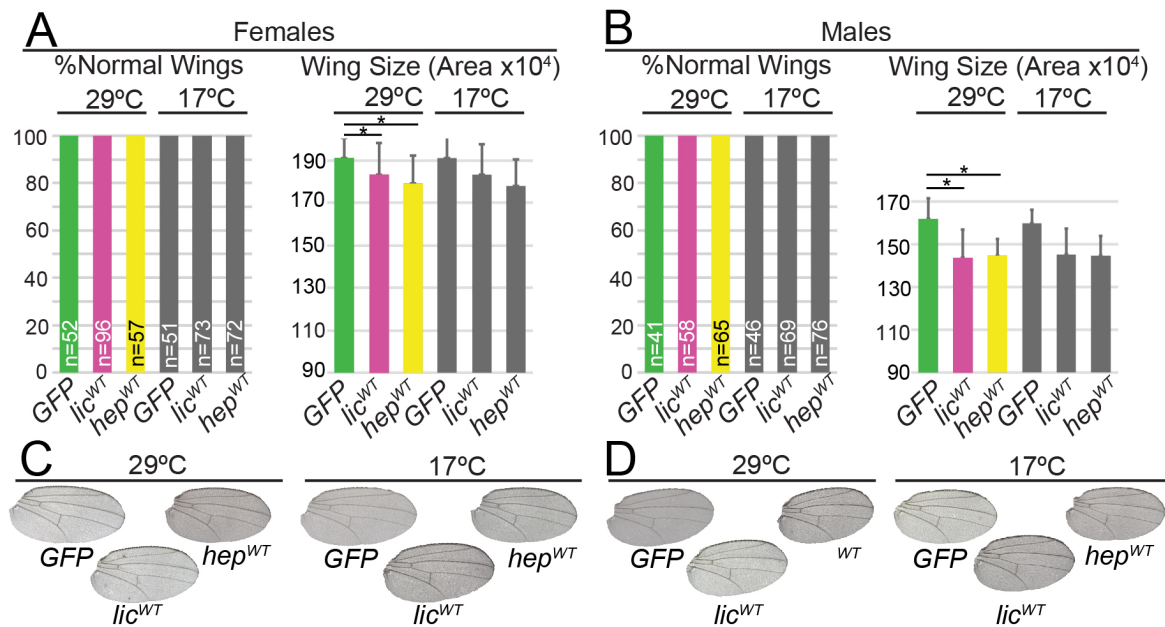


Figure S3. Control wing analysis of transgene expression in the absence of genetic ablation. The *nub-Gal4 UAS-GFP*, *nub-Gal4 UAS-lic^{WT}* and *nub-Gal4 UAS-hep^{WT}* genotypes were kept at the same temperature as those in Figures 3 and 4. (A-B) Percentage of regenerated wings and wing areas, as an indication of wing size, in females and males. Colored bars correspond to the experiments with a temperature shift to 29°C. Gray bars correspond to the experiments performed at 17°C. *p < 0.05. (C-D) Examples of wings obtained from the indicated genotypes and temperatures.

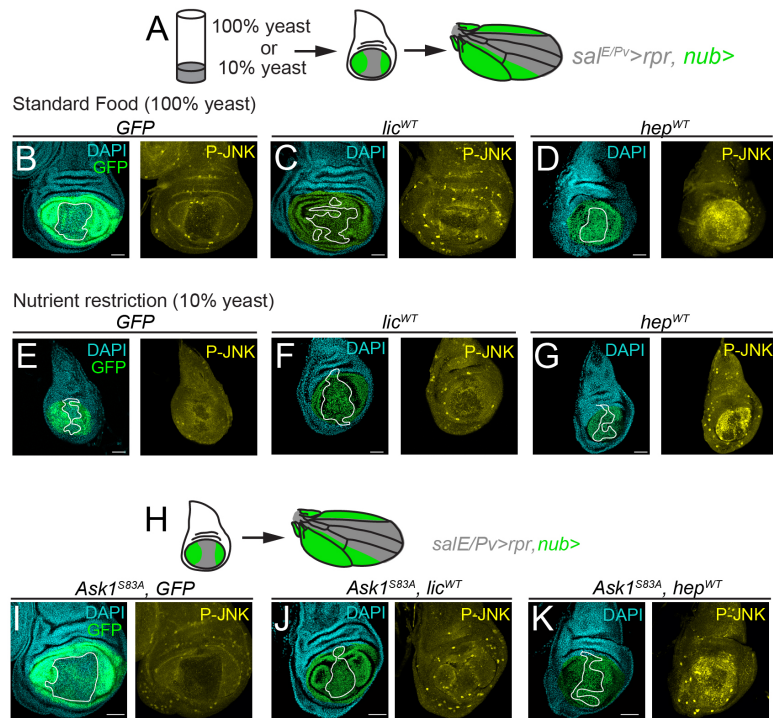


Figure S4. Anti-P-JNK antibody staining corresponding to the experiments in Figures 3 and 4. (A) Scheme (top) of the zones of dual transactivation in the wing disc and its corresponding zone in the adult wing in conditions of standard food or nutrient restriction. Gray, apoptotic zone generated by *sal^{E/Pv}>rpr*, green, transgene activation under *nub-Gal4* (*nub>*) to drive the expression of the indicated UAS transgene. Three UAS transgenes were used: *UAS-GFP*, as a control; *UAS-lic^{WT}*, to express the MAPKK *licorne*; and *UAS-hep^{WT}*, to express the JNKK *hemipterous*. (B-D) Anti-P-JNK antibody staining of *nub>GFP* (B), *nub>lic^{WT}* (C) and *nub>hep^{WT}* (D) wing discs in standard food conditions. (E-G) Anti-P-JNK antibody staining of *nub>GFP* (E), *nub>lic^{WT}* (F) and *nub>hep^{WT}* (G) wing discs in conditions of nutrient restriction. (H) Scheme (top) of the zones of dual transactivation in the wing disc and their corresponding zones in the adult wing. Gray, apoptotic zone (*sal^{E/Pv}>rpr*); green, zone of transgene activation under the *nub-Gal4* (*nub>*) driver. Three UAS transgenes were expressed together with the Ser83 mutant construct *UAS-Ask1^{S83A}* in standard food conditions: *UAS-GFP*, as a control; *UAS-lic^{WT}*, to express *licorne*; and *UAS-hep^{WT}*, to express *hemipterous*. (I-K) Anti-P-JNK antibody staining of *nub>Ask1^{S83A}, GFP* (I), *nub>Ask1^{S83A}, lic^{WT}* (J) and *nub>Ask1^{S83A}, hep^{WT}* (K) wing discs. The white line in the confocal images outlines the zone of apoptotic cells indicated by the pyknotic nuclei. Nuclei were stained with TO-PRO-3 (TP-3). *UAS-GFP* (*GFP*) was used to monitor the *nub>* zone. In (C), (D), (F), (G), (J) and (K), the *nub>* zone is colored in green. Scale bars: 50 μ m.

Chapter 3

*“Me sentí como un insecto absurdo en una noche ventosa,
adherido a un alto muro, **sin razones, sin planes, sin creencias**”*

Haruki Murakami, Sputnik, mi amor, 1999

ROS-mediated TNFR Wengen activation in response to apoptosis

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Abstract

Tumor necrosis factor receptors (TNFR) control pleiotropic pro-inflammatory functions ranging from apoptosis to survival. The ability to trigger a particular function will depend on the upstream activation, association with regulatory complexes, and downstream pathways. In *Drosophila*, two TNFRs have been identified, Wengen (Wgn) and Grindelwald (Grnd). Although several reports associate these receptors with JNK-dependent apoptosis, it has recently been found that Wgn activates a variety of functions. We demonstrate that Wgn is required for survival by protecting cells from apoptosis. This is mediated by dTRAF1 and results in the activation of p38 MAP kinase. Remarkably, Wgn is required for apoptosis-induced regeneration and is activated by the reactive oxygen species (ROS) produced following apoptosis. This ROS activation is exclusive for Wgn, but not for Grnd, and can occur after knocking down Eiger/TNF α . The extracellular Cys-rich domain of Grnd is much more divergent than that of Wgn, which is more similar to TNFRs from other animals, including humans. Our results show a novel TNFR function that responds to stressors by ensuring the cell survival required for regeneration.

Keywords: Apoptosis, Cell Signaling, Cytokines, Oxidative stress, Regeneration.

Introduction

The cytokine tumor necrosis factor alpha (TNF α) is rapidly released after trauma, injury or infection and acts as a central mediator in the inflammatory response. It signals through the TNF receptors (TNFRs) and its function is currently understood to be pleiotropic, playing a role in apoptosis, cell survival, and cell proliferation, the outcome of which will be determined by different TNFRs and complex signaling networks (Gough and Myles, 2020).

Oxidative stress, generated by the production of reactive oxygen species (ROS), is recognized as an underlying cause of a variety of inflammatory diseases (Conner & Grisham, 1996; Droge, 2002; Finkel, 2011). ROS produce deleterious effects on cells because of their ability to oxidize cell structures, such as DNA (Van Houten et al., 2018), membrane lipids and proteins (Colquhoun, 2010; Moriarty-Craige & Jones, 2004). However, it is increasingly well-accepted that ROS-induced post-translational modifications of proteins may also be of physiological relevance in cell signaling. Among these is the oxidation of cysteine residues in receptor proteins, which results in their activation (Lipton et al., 2002; Truong & Carroll, 2012). Likewise, oxidative stress can modify the reduced thiol groups of the TNFR1 extracellular cysteine-rich domain (CRD), which is the hallmark domain shared by the TNFR superfamily (Dominici et al., 2004). This raises the intriguing possibility that oxidative stress might modulate TNFR signaling. Indeed, oxidative stress promotes the self-association of the subunits of mammalian TNFR1 and 2, which results in ligand-independent signaling as well as enhanced ligand-dependent TNF signaling (Ozsoy et al., 2008).

Tolerable levels of ROS can propagate as paracrine signals and modulate the intracellular machinery that will reconstruct the damaged tissues during regeneration (Brock et al., 2017; Diwanji & Bergmann, 2018; Farrell et al., 2022; Fogarty et al., 2016; Khan et al., 2017; Patel et al., 2019; Pérez et al., 2017; Santabárbara-Ruiz et al., 2015; Serras, 2022; Weavers et al., 2019). In the presence of ROS, thioredoxin dissociates from the MAP3 kinase Apoptosis signal-regulating kinase 1 (Ask1), following which Ask1 oligomerizes, autophosphorylates, recruits its partners and forms an active kinase complex (Bunkoczi et al., 2007; Matsuzawa, 2016; Obsil & Obsilova, 2017; Takeda et al., 2008). An active Ask1 catalytic domain triggers the phosphorylation of the MAP2 kinases that in turn phosphorylate JNK and p38 to induce apoptosis (Bunkoczi et al., 2007; Ichijo et al., 1997; Tobiume et al., 2001). But Ask1 has other functions beyond apoptosis. In *Drosophila*, Ask1 operates in the gut and imaginal disc epithelia as a survival signal in tissue regeneration by triggering a non-apoptotic function of p38 (Patel et al., 2019; Santabárbara-Ruiz et al., 2019). This is achieved by a ROS-dependent, Akt-induced phosphorylation of Ser83 in Ask1 (Ser174 in humans) that is essential to redirect the Ask1 signaling pathway towards p38 and thus drive regeneration (Esteban-Collado et al., 2021).

The kinase activity of Ask1 is also stimulated by TNF α via members of the TNF Receptor-Associated Factor (TRAF) family of adaptor proteins (Ichijo et al., 1997; Nishitoh et al., 1998). Upon the binding of TNF α to TNFRs, a subsequent interaction with TRAFs occurs, facilitating the transduction of the signal to Ask1 and thereby modulating its activity (Hoeflich et al., 1999; Nishitoh et al., 1998; Obsil & Obsilova, 2017; Shiizaki et al., 2013). How this TNF/TNFR/TRAF axis drives apoptosis or survival is poorly understood. In contrast with the large families of both TNF and TNFRs in mammals, *Drosophila* has only one TNF α ortholog, *eiger* (*egr*), and two TNFRs, *wengen* (*wgn*) and *grindelwald* (*grnd*). Grnd mediates the pro-apoptotic function of Egr/TNF α , and its overexpression activates JNK-dependent apoptosis (Andersen et al., 2015; Palmerini et al., 2021). Wengen was first discovered as a TNFR that is able to bind to TRAF, trigger JNK-dependent apoptosis, and transduce signals from Egr/TNF α (Geuking et al., 2005; Kanda et al., 2002; Kaupilla et al., 2003). However, it has been shown

that while the knockdown of *grnd* blocks apoptosis, that of *wgn* does not (Andersen et al., 2015), suggesting that *wgn* and *grnd* are not redundant. Moreover, both TNFRs are transmembrane proteins that form hexamers for ligand-joining, but Wgn binds to Egr/TNF α at an affinity that is three orders of magnitude lower than Grnd (Palmerini et al., 2021). Recent work has demonstrated that in the gut, Wgn suppresses dTRAF3-mediated lipolysis independently of its ligand and maintains tissue homeostasis, while Wgn-dTRAF2-mediated immune suppression occurs in an Egr-dependent manner (Loudhaief et al., 2023). In addition, the ligand-independent function of Wgn has been recently demonstrated to associate with unrelated factors such as FGFR to regulate vesicle trafficking during tracheal development (Letizia et al., 2023). Wgn is also expressed in photoreceptor progenitors and binds to moesin for axonal pathfinding in a ligand-independent manner (Ruan et al., 2013). Moreover, *Drosophila* TNFRs show a different subcellular localization. Grnd is mainly found on the apical side of epithelial cells and becomes internalized upon binding to Egr, whereas Wgn is mainly found in intracellular vesicles (Andersen et al., 2015; Letizia et al., 2023; Loudhaief et al., 2023). So far, the evolutionary origin of the two *Drosophila* TNFRs and their relationship to TNFRs from other species have not been investigated in detail, limiting our understanding of how the contrasting functions and molecular behavior of these two receptors may have originated.

Therefore, we used the wing imaginal disc epithelium to explore whether these *Drosophila* TNFRs are involved in survival or apoptosis, whether they respond to oxidative stress and, ultimately, whether they are required for apoptosis-induced regeneration. Here we show that Wgn/dTRAF1 is required for cell survival, in contrast to the apoptotic role of Grnd/dTRAF2. Evolutionary analyses of TNFRs showed an independent and ancient origin of *grnd* and *wgn*, reinforcing the idea of a subfunctionalization of these genes and a higher degree of similarity between the CRDs of Wgn and the TNFRs of humans and other deuterostomes. Indeed, Wgn, but not Grnd, is required for regeneration and for the activation of p38 in the damage response. Interestingly, the activation of Wgn is sensitive to the ROS produced by damaged cells and is not affected by knocking down Egr/TNF α .

Results

Wgn is required for survival by protecting cells from apoptosis

We first studied the involvement of the TNFRs Grnd and Wgn in apoptosis and survival. The two *Drosophila* TNFRs are normally expressed in the wing imaginal discs (Palmerini et al., 2021), whereas Eiger/TNF α (*egr*) is not. Ectopic expression of *egr* in *Drosophila* imaginal discs results in JNK-dependent apoptosis (Brodsky et al., 2004; Igaki et al., 2002; Moreno, Yan, et al., 2002). We ectopically expressed *egr* in the wing disc, using the *Gal4/UAS/Gal80^{TS}* transactivation system, and simultaneously knocked down the TNFRs using appropriate RNAi strains. We used the *hedgehog-Gal4* strain (hereafter *hh>*) to activate the transcription of *egr* in the posterior compartment using the *UAS-egr^{weak}* strain (hereafter *egr^{weak}*), a transgene that causes mild/moderate *egr* overexpression (Moreno, Basler, et al., 2002).

The expression of *egr^{weak}* resulted in low levels of apoptotic cells in the posterior compartment of the disc (Figs. 1A,I, and EV1A). However, *egr^{weak}* expression resulted in a strong abolition of apoptosis when *grnd* was downregulated (Figs. 1B,I, and EV1F), which coincides with previous findings that Grnd promotes JNK-dependent apoptosis (Andersen et al., 2015; Palmerini et al., 2021). In contrast, *egr^{weak}* expression resulted in a strong enhancement of apoptosis when *wgn* was downregulated (Fig. 1E, I). This observation was corroborated with an independent *wgn RNAi* strain targeting a different region in the coding sequence (Fig. EV1D,E).

Remarkably, expression of any of the two *wgn* RNAi strains alone under the *hh*> driver produced only few scattered caspase positive cells (Fig. EV1D,E). Therefore, the massive *wgn*-related apoptosis occurs only in stressed conditions generated by *egr* expression, suggesting that *wgn* is recruited for a stress-dependent environment.

Next, we knocked down the adaptor protein *dTRAF2* and the MAP3 kinase *Tak1* and found reduced or similar levels of apoptosis compared to *egr^{weak}* alone, respectively (Figs. 1C,D, I, and EV1G,H), confirming the role of those genes in mediating the pro-apoptotic function of TNF signaling. In contrast, knocking down *dTRAF1* and *Ask1* resulted in increased apoptosis in comparison to *egr^{weak}* alone (Figs. 1F,G,I and EV1I, J). Note that when *dTRAF2* or *grnd* is downregulated in *egr^{weak}* cells, the cell death area ratio is slightly lower than *egr^{weak}* alone (Fig. 1I), confirming that dTRAF2 and Grnd contribute to apoptosis in *egr^{weak}* cells. All these observations were corroborated with independent RNAi strains from different sources (Figs. 1I and EV1).

To check for epistasis between *grnd* and *wgn*, we activated *hh*> *egr^{weak}* and knocked down both TNFRs. We found high levels of cell death compared to *wgn* RNAi alone (Fig. 1H, I), which demonstrates that *wgn* downregulation is dominant over *grnd*. This is surprising, as it is generally assumed that Egr interacts with Grnd to induce apoptosis via JNK, which in turn activates the proapoptotic gene *hid* (Andersen et al., 2015; Diwanji & Bergmann, 2020; Fogarty et al., 2016; Igaki et al., 2002; Moreno, Yan, et al., 2002; Sanchez et al., 2019; Shlevkov & Morata, 2012). Interestingly, Egr is necessary for the stabilization of the pro-apoptotic gene *hid* and can regulate HID-induced apoptosis independently of JNK (Shklover et al., 2015). Therefore, *egr^{weak}* cells in which *grnd* and *wgn* are downregulated can still be eliminated, due to both the lack of the Wgn survival signal and the ability of the pro-apoptotic Grnd/JNK signal to trigger an alternative apoptosis pathway.

These results show that *wgn* is required in cell survival, likely through the Wgn/DTRAF1/Ask1 signaling cassette. In addition, as *grnd* has been demonstrated to be involved in JNK-dependent apoptosis (Andersen et al., 2015), our observations also indicate that the functions of *wgn* and *grnd* are essentially different.

Wgn and Grnd are phylogenetically divergent

Opposing roles between proteins of the TNFR superfamily suggests that they have an ancient origin and have followed divergent evolutionary paths. To track the differences observed between *grnd* and *wgn*, we decided to investigate the evolutionary origin of these two *Drosophila* genes. This involved searching for homologs in other animal lineages and performing maximum likelihood (ML) phylogenetic analyses using the amino acid sequences of the CRDs, the only conserved protein domain shared by all TNFRs (Fig. 2A and EV22A). We observed that *Drosophila* Grnd and Wgn CRD sequences were in separate branches of the tree, in two different, highly supported monophyletic groups that together included most of the TNFRs genes we had identified in arthropods (Supplementary Table 1, Fig. EV2B). The Wgn branch included genes from multiple arthropod groups, ranging from dipterans such as *Drosophila* to chelicerates, indicating that Wgn has a very ancient origin that predates the diversification of crown arthropods before the Cambrian, ~546 million years ago (mya) (Lozano-Fernandez et al., 2020) (Fig. 2B). Grnd had a more restricted taxonomic distribution and was only present in pancrustacean species (insects plus crustaceans), suggesting that this gene family had a slightly younger origin (emerging at least 514 mya) (Wolfe et al., 2016) (Fig. 2B). Thus, these results showed that Grnd and Wgn are very different from an evolutionary perspective, suggesting they originated through an ancient duplication event of an ancestral TNFR in pancrustaceans followed by a highly asymmetric evolution (Holland et al., 2017) or

even that there is an independent origin of the *grnd* and *wgn* CRDs (i.e. through independent recruitment/exon shuffling with cysteine-rich regions from other gene families). Consistent with this, Grnd and Wgn clades were more closely related to some of the TNFR gene families from deuterostome species than they were to each other (Fig. 2C and Fig. EV2C). However, in this case the bootstrap supports were too low to reliably assign orthology relationships, probably because CRD sequences are too divergent and short (less than 60 aa) to robustly establish evolutionary relationships between TNFR genes from distant animal phyla (i.e. human and insects) (Huang et al., 2008).

Therefore, we applied an alternative approach to assess similarities between the deuterostome TNFRs, Grnd, and Wgn and performed a PCA-based alignment (Fig. 2C) and a pairwise BLAST comparison (Fig. EV2C). In both analyses, Grnd CRD sequences clustered separate from all other TNFRs, while Wgn family members exhibited a higher degree of similarity with deuterostome TNFRs, clustering together with some of the CRD sequences from hemichordates (the acorn worm, *Saccoglossus kowalevskii*), non-vertebrate chordates (the European amphioxus, *Branchiostoma lanceolatum*), and vertebrates (*Homo sapiens*). Thus, Grnd would constitute a more divergent member of the TNFR superfamily than Wgn, a notion also supported by the unique and derived pattern of cysteine residues that characterize Grnd CRDs, which contain 8 conserved cysteines instead of the 6 cysteines typically found in most TNFR families (Fig. EV2D) (Palmerini et al., 2021). These results reinforce the hypothesis of the repurposing of *Drosophila grnd* and *wgn* for different biological functions. The expansion and subfunctionalization of TNFR for apoptosis, survival, and the stress response has been suggested for other metazoan clades (Quistad & Traylor-Knowles, 2016).

The protective role of Wgn is mediated by the activation of the p38 MAP kinase

Next, we analyzed the function of Wgn in cell survival. Although Ask1 can activate JNK and p38 (Tobium et al., 2001), stimulation of p38 and inhibition of the JNK-proapoptotic function is necessary for a regenerative response to damage (Patel et al., 2019; Santabábara-Ruiz et al., 2019). Therefore, we hypothesized that the survival role driven by Wgn is likely accomplished by p38 rather than JNK.

To explore this hypothesis, we tested if the apoptosis induced by knocking down *wgn* can be abolished by ectopic stimulation of JNK or p38. We used *hh>egr^{weak}*, *wgn RNAi*, which exacerbated apoptosis, and expressed the MAP2 kinases (*UAS-hep^{WT}* or *UAS-lic^{WT}*) upstream of either JNK or p38. An ectopic activation of the JNK pathway by *UAS-hep^{WT}* resulted in increased levels of apoptosis in comparison to the control (*wgn RNAi* alone) (Fig. 3A, C, D). In contrast, an ectopic activation of p38 by *lic^{WT}* resulted in a strong decrease in apoptosis (Fig. 3A, B, D), suggesting that activated p38 functions downstream of Wgn for cell survival.

Additionally, p38 RNAi exacerbates apoptosis in *egr^{weak}* cells (Fig. EV3A). While *lic^{WT}* alone results in few scattered apoptotic cells (Esteban-Collado et al., 2021), expression of *lic^{WT}* in *egr^{weak}* cells induced extensive apoptosis (Fig. EV3B), possibly due to the excess of active p38 generated by the addition of phospho-p38 in response to *egr^{weak}* and the activity of *lic^{WT}*. These observations support that the survival role of p38 must be finely controlled, as an excess or shortage of p38 activity is detrimental to the tissue (Esteban-Collado et al., 2021).

Wgn is activated by ROS

Next, we investigated whether Wgn responds to the stress generated by the expression of *egr*. To this end, we first examined the localization of Wgn and Grnd after *egr^{weak}* expression. It has been reported that the majority of Wgn is localized in the cytoplasm in many organs, likely in intracellular vesicles rather than at the plasma membrane (Letizia et al., 2023; Loudhaief et al.,

2023; Palmerini et al., 2021). By contrast, Grnd is localized in the plasma membrane, making it more accessible to Egr/TNF α (Palmerini et al., 2021). We confirmed these localization patterns for Wgn and Grnd in control imaginal discs (Fig. 4A and B).

After ectopic *egr^{weak}* expression in the posterior compartment, Wgn particles were found accumulated in the cytoplasm of the anterior compartment, particularly in anterior cells close to the border with the posterior compartment, and they were absent in the *egr^{weak}* posterior compartment (Fig. 4C). Apoptotic cells in the latter compartment (*egr^{weak}*) are characterized by pyknotic nuclei and are positive for Dcp1. These cells can be found along the apical-basal axis, although they eventually tend to concentrate on the basal side of the epithelium (Fig. EV4A,B). An accumulation of Wgn was observed in healthy anterior cells adjacent to both apical and basal *egr^{weak}* cells (Fig. 4C, and Fig. EV4A,B). By contrast, Grnd was maintained on the apical membrane of the anterior compartment and was found internalized in the *egr^{weak}* posterior compartment (Fig. 4D), which aligns with previous observations that Grnd is translocated from the membrane after binding to Egr/TNF α (Andersen et al., 2015).

It is known that apoptosis generates oxidative stress due to the production of ROS of mitochondrial origin that can propagate to recruit neighboring cells for damage repair (Serras, 2022). Furthermore, the oxidation of the TNFR CRD by ROS is a physiological mechanism able to transduce the signal (Ozsoy et al., 2008). Thus, to investigate the molecular mechanism underlying the accumulation of Wgn in neighboring cells after *egr* expression, we first checked if ROS were produced after *hh>egr^{weak}*. We used in vivo imaging with the cell-permeant fluorogenic probe MitoSOX, which is non-fluorescent in the reduced state and exhibits bright fluorescence upon oxidation by mitochondrial superoxide. MitoSOX-positive cells were found in the *egr^{weak}* compartment and they co-localized with cells positive for TO-PRO-3, a stain that is very sensitive to dead and dying cells (Fig. 4E). MitoSOX co-localization with TO-PRO-3 cells was also detected in *egr^{weak}* cells after knocking down *wgn*, indicating that inhibition of *wgn* does not block ROS production (Fig. EV4C). To discern whether ROS production responds to *egr^{weak}* expression or to apoptosis, we co-expressed *egr^{weak}* with the baculovirus protein p35, which blocks the effector caspases (Hay & de Belleruche, 1994). We found that neither MitoSOX nor TO-PRO-3 were detected when apoptosis was blocked in *egr^{weak}*-expressing cells (Fig. 4F). This suggests that the accumulation of ROS is caused by apoptosis rather than the expression of *egr*.

Next, we enzymatically decreased ROS production using ectopic expression of the ROS scavengers *Superoxide dismutase 1* and *Catalase* (*UAS-Sod1:UAS-Cat*). This resulted in a reduction of ROS (Fig. 4G) and the accumulation of Wgn particles near the ablated area compared to *egr^{weak}* alone (Fig. 4H). By contrast, Grnd localization was not altered following ROS depletion (Fig. 4I).

It is worth noting that *egr^{weak}* cells induce the phosphorylation of p38 in neighboring cells (Fig. EV4D), as occurs in cells that do not express the pro-apoptotic gene *reaper* (*rpr*) (Santab  bara-Ruiz et al., 2015). ROS depletion and p35 expression reduce phospho-p38 levels (Fig. EV4E,F). This suggests that p38 activation depends on ROS generated by apoptotic *egr^{weak}* cells.

Since the expression of the *egr^{weak}* results in apoptosis, we decided to induce apoptosis in an Egr-independent manner to monitor the localization of both TNFRs. With this aim, we expressed *rpr* using the *sal^{E/Pv}-Gal4* driver, whose expression is restricted to the central part of the wing imaginal disc (henceforth *sal^{E/Pv}>*). As a result, Wgn accumulated in the cells surrounding the apoptotic zone (Fig. 5A,G and EV5A). Contrastingly, apical Grnd localization in the tissue surrounding the apoptotic zone did not vary (Fig. 5B,H). Wgn and Grnd were also detected in cellular debris in the apoptotic *rpr* expression zone. Moreover, we also reduced ROS

production in the *rpr*-ablated region (*sal^{E/Pv}>rpr*, *Sod1:Cat*) and found a decrease in Wgn, but not Grnd, levels in the cells surrounding the ablated zone (Fig. 5C, D, G, H and EV5B).

In addition, we found that *egr/TNFα* is autonomously expressed after inducing apoptosis by *rpr* (Fig. EV5D, E). To test if the effects on Wgn localization were due to ROS or to the expression of *egr*, we used RNAi to knock down *egr* in the apoptotic cells and found that Wgn accumulation was not altered by knocking down *Egr/TNFα* (Fig. 5E, F, G, H, and EV5C).

Together, these observations suggest that the Wgn response to apoptotic ROS production occurs independently of a reduction in *Egr/TNFα*.

Wgn, but not Grnd, is necessary for p38-dependent regeneration

Regeneration in the gut and in imaginal discs depends on p38 in a ROS-dependent manner (Patel et al., 2019; Santabábara-Ruiz et al., 2019). Therefore, we wondered whether Wgn is necessary for p38-dependent regeneration. We used a double transactivation system to simultaneously induce apoptosis in one domain of the wing disc to stimulate regeneration and to knock down either *wgn* or *grnd* in adjacent regenerating cells (Fig. 6A). We first confirmed that the mutants for *wgn* and *grnd* used in this work do not affect normal growth and patterns (Fig. EV6A). However, knocking down *wgn* after inducing apoptosis resulted in anomalous wings, a characteristic of incomplete regeneration (Fig. 6B). Most of these wings showed a reduced size and defective patterns of veins and interveins. By contrast, wings carrying a knock down of *grnd* did not show a reduced size or defects in wing patterning (Fig. 6B), suggesting that regeneration of the apoptotic zone was completed even in the absence of *grnd*. To confirm this observation, we induced cell death (*sal^{E/Pv}>rpr*) in an independent *grnd* mutant, *grnd^{minos}*, in homozygosis and heterozygosis and found that wings regenerated normally. This result demonstrates that *wgn*, but not *grnd*, is necessary for the regenerative response after apoptosis.

Next, to test if *wgn* is required for p38-driven regeneration, we analyzed if phosphorylated p38 is affected by *wgn* downregulation after inducing apoptosis. We used the double transactivation system to induce apoptosis in the *sal*-central zone of the wing pouch and to knock down *wgn* or *grnd* in the wing pouch (henceforth *nub>*). Intact discs show low levels of phosphorylated p38 (Fig. 6C). In apoptosis-induced discs, we detected activation of p38 in the wing pouch surrounding the apoptotic cells (Fig. 6CD), as previously described (Esteban-Collado et al., 2021; Santabábara-Ruiz et al., 2015, 2019). Likewise, phosphorylation of p38 in cells near the apoptotic zone was found after knocking down *grnd* (Fig. 6E). However, after knocking down *wgn*, p38 phosphorylation in cells surrounding the apoptotic zone was strongly reduced (Fig. 6F). The accumulation of pyknotic nuclei after ectopic *rpr* expression indicates that apoptosis is not suppressed after knocking down *grnd* or *wgn*. As for the *ap/ci* drivers (Fig. 6B), the resulting *sal^{E/Pv}>rpr*, *nub> wgn RNAi* adult wings showed severe anomalies, indicating impaired regeneration (Fig. EV6B). These results demonstrate that *wgn*, but not *grnd*, is key for the activation of p38 signaling during the regenerative response to apoptosis. Remarkably, *wgn* inactivation in the same cells expressing *rpr* did not show significant defects, suggesting that *wgn* is required non-autonomously for regeneration (Fig. EV6C).

Discussion

In this work we have demonstrated that the conserved TNFR Wgn is activated by oxidative stress, accumulates near the source of ROS, and confers survival to cells even after knocking down *Egr/TNFα*. This function primarily involves exacerbating p38 activity, likely via dTRAF1/Ask1, and is tightly linked to the oxidative stress induced following cell damage.

Mammalian TNFR pathways have pleiotropic functions as a result of complex regulatory mechanisms (Gough & Myles, 2020). Upon binding to TNF α , a core signaling complex is constructed on the cytoplasmic tail of the TNFRs. This signaling complex includes the TRAF adaptor proteins as major signal transducers for the TNFRs. In mammals and *Drosophila*, a range of biological functions, such as adaptive and innate immunity, embryonic development, and stress response, are mediated by TNFRs/TRAFs through the induction of cell survival, proliferation, differentiation, and/or cell death (Colombani & Andersen, 2023). Thus, TRAFs add complexity to the upstream TNFR and consequently to the signal transduction (Chung et al., 2002). In addition to TNFRs, TRAFs associate to MAP3 kinases such as Ask1 or Tak1, which will trigger the p38 or JNK MAP kinase pathways (Hoeflich et al., 1999; Nishitoh et al., 1998). In mammals, once TNFR1 is activated and binding has occurred with the core complex, typically comprised of TRAF2, TRAF5 and the receptor-interacting kinase RIPK1, post-translational modifications will determine its ability to activate p38 or JNK for survival and inflammation (Dostert et al., 2019). If these protein modifications are disrupted, the signaling complex triggers apoptosis (Dostert et al., 2019; Vince et al., 2009). Wgn signaling in *Drosophila* is also pleiotropic and its function in the cell will also depend on upstream and downstream signals. Wgn was first described as pro-apoptotic after binding to Egr/TNF α (Kanda et al., 2002), with dTRAF2 or dTRAF1 interacting with the TNFR (Geuking et al., 2005; Kauppila et al., 2003). However, the lack of the death domain, distinctive of many TNFRs, and the inability of *wgn* mutants to rescue the apoptotic phenotype generated by Egr/TNF α , suggests that apoptosis is not the main function of *wgn* (Andersen et al., 2015; Kanda et al., 2002). Indeed, Wgn is emerging as a pro-survival TNFR and, as shown in mammals, the combination of TRAFs or other adapter proteins in the C-terminal core complex could divert the pathway towards functions other than apoptosis. Indeed, our regeneration assay, where *Drosophila* Wgn-dTRAF1 has a survival function, as well as where Wgn-dTRAF3 suppresses lipolysis in the gut, are examples of a *Drosophila* TNFR acting independently of Egr/TNF α (Loudhaief et al., 2023). In contrast, Grnd/dTRAF2 drives the pathway towards JNK-driven apoptosis in a ligand-dependent manner, likely through the Tak1 MAP3 kinase (Andersen et al., 2015; Palmerini et al., 2021). Furthermore, Grnd displays nanomolar binding affinity for Egr that is three orders of magnitude higher than it is for Wgn, suggesting that canonical Egr signaling in *Drosophila* occurs predominantly through Grnd activation rather than through Wgn (Palmerini et al., 2021).

The TNFR superfamily has been described as containing a CRD on their N-terminal. Cysteine residues have active thiols that can be efficiently oxidized by ROS, a physiological mechanism to transmit external signals to the intracellular system (Gotoh & Cooper, 1998; Kamata et al., 2005; Zhang et al., 2001) and subsequently alter protein structure, interaction with partners, and subcellular localization (Sies et al., 2017). Moreover, human TNFR1/2 can suffer oxidative stress-induced self-association due to modifications of the CRD, resulting in ligand-independent signaling (Ozsoy et al., 2008). Therefore, we speculate that, similarly to mammals, the oxidation of Wgn could promote its self-association and influence the partner preferences of the core signaling complex, i.e., Wgn-dTRAF1, in a ligand-independent manner. In addition, dTRAF1 has been described to be a positive interaction partner of Ask1 (Kuranaga et al., 2002). Hence, we propose that Wgn-dTRAF1-Ask1 is a signaling module activated upon oxidative stress to ensure survival in cells that will be involved in the regenerative response. Moreover, we recently demonstrated that Ask1 requires the activity of the nutrient-dependent Pi3K/Akt signal to divert Ask1 function to p38 phosphorylation in cell survival and regeneration (Esteban-Collado et al., 2021). Thus, we conclude that it is not only Pi3K/Akt, but also Wgn, that will be necessary for leading Ask1 to induce a p38 response to apoptosis.

Here, we propose a model for the targets that respond to ROS upon cell damage. Cells that have been damaged, either by injuries or apoptosis, produce ROS, normally of mitochondrial origin (Murphy, 2009). ROS spreads from damaged or dying cells to the nearby healthy cells, acting as early signals for tissue recovery in different organisms such as flatworms and mammals (Gauron et al., 2013; Rampon et al., 2018). In our model, the ROS-dependent post-translational modifications will primarily target three branches that converge with Ask1 (Fig. 7). First, we have the thioredoxin bound to the inactive Ask1 signaling complex, which upon oxidation will be dissociated and allow the active Ask1 to interact with TRAFs (Matsuzawa, 2016; Nishida et al., 2017; Sakauchi et al., 2017; Shiizaki et al., 2013; Tobiume et al., 2001). Second, there is the insulin/Pi3K/Akt signaling pathway that will phosphorylate Ask1 to reroute it for survival and ultimately phosphorylation of p38 (Esteban-Collado et al., 2021; Kim et al., 2001; Santab rbara-Ruiz et al., 2019). Third, because we have found that the Wgn/dTRAF1/Ask1 axis is involved in survival as a stress response, we hypothesize that Wgn oxidation could be a signal that contributes to the interaction of dTRAF1 with the Ask1 core signaling complex (Noguchi et al., 2005). Thus, the divergent role of Grnd and Wgn is driven not only by their different affinity for the ligand Egr/TNF , but also by the ROS-dependent activation of Wgn. We have shown here that Wgn accumulation near the damaged zone can be reverted after ROS depletion, indicating that ROS produced by dying cells is involved in the Wgn response after damage. Lineage experiments have shown that these cells near the damaged zone are responsible for most of the regenerated epithelium (Bosch et al., 2008; Repiso et al., 2013). However, we cannot rule out that the accumulation of Wgn near the affected area responds not only to a reorganization of vesicles, but also to a transcriptional response. Indeed, RNAseq of regenerating imaginal discs has shown that *wgn* is transcriptionally upregulated in the earliest phase of regeneration (Vizcaya-Molina et al., 2018).

We observed a very high degree of divergence between Grnd and Wgn, showing that both gene families are of extremely ancient origin and are found across pancrustacean and arthropod lineages, respectively. This taxonomic distribution suggests that Grnd originated through a duplication and fast divergence at the base of the pancrustacean phylogenetic tree. These results match the subfunctionalization observed in both genes, with Grnd having a pro-apoptotic function and Wgn promoting cell survival. The amplification and subfunctionalization of TNFRs has also been observed in different lineages for adaptation to biotic or abiotic stress (Quistad & Traylor-Knowles, 2016).

Remarkably, we observed that there is also a very high sequence divergence between the CRDs of Grnd and those of all the other TNFR families we studied. By contrast, Wgn CRDs were much more canonical, and we found that they clustered together with the CRDs from deuterostome TNFRs, including those from humans.

We speculate that the subcellular location of Wgn and Grnd may contribute to the different functions of both receptors. Grnd is more exposed at the apical side of the plasma membrane, which makes this receptor more accessible for ligand interactions (Palmerini et al., 2021) to transduce a JNK-dependent apoptotic signal (Moreno, Yan, et al., 2002; Shlevkov & Morata, 2012; Tobiume et al., 2001). Wgn, embedded in cytoplasmic vesicles, is less accessible to the ligand and could be more restricted to being activated by local sources of signaling molecules, such as ROS. In contrast to initial reports (Kanda et al., 2002; Kauppila et al., 2003), *wgn* loss of function does not rescue Egr-induced apoptosis in the *Drosophila* eye (Andersen et al., 2015), which supports our observation in the wing that Wgn is not required for Egr-induced apoptosis. Instead, Egr-induced apoptosis generates ROS, which target intracellular Wgn to foster a cell survival program in cells adjacent to the apoptotic zone.

Disclosure and competing interests statement

The authors declare no competing interests.

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Materials and methods

Drosophila strains

Animals were reared on standard fly food. The *sal^{E/Pv}-LHG* and *lexO-rpr* *Drosophila* strains have been previously described (Santabàrbara-Ruiz et al., 2015). Other strains were: *UAS-egr^{weak}* (Moreno, Basler, et al., 2002), *UAS-lic^{WT1.1}* (Terriente-Félix et al., 2017), *UAS-hep^{WT}* (Uhlirva & Bohmann, 2006), *sal^{E/Pv}-Gal4* (Barrio & de Celis, 2004), *ci-Gal4* (Martin & Morata, 2006), *UAS-eiger-RNAi* (Kuranaga et al., 2002). The *eiger-lacZ* strain was provided by K. Basler. The *w¹¹⁸;+;+* strain was used as a control. These strains are described in FlyBase: *ap-Gal4*, *nub-Gal4*. From the Bloomington *Drosophila* Stock Center (BDSC): *ptc-Gal4* (RRID:BDSC_2017), *tub-Gal80^{TS}* (RRID:BDSC_7017), *UAS-rpr* (RRID:BDSC_92781), *UAS-Sod1.a* (RRID:BDSC_24754), *UAS-Cat.a* (RRID:BDSC_24621), *UAS-p35* (RRID:BDSC_5072), *UAS-wgn-RNAi* (TRiP, RRID:BDSC_55275), *UAS-Ask1-RNAi* (RRID:BDSC_35331; TRIP1 in figures), *UAS-GFP* (RRID:BDSC_4776), *UAS-Tak1-RNAi* (RRID:BDSC_53377), *UAS-dTRAF2-RNAi* (6) (RRID:BDSC_33931), *UAS-dTRAF1-RNAi* (4) (RRID:BDSC_55226), *UAS-Ask1* (RRID:BDSC_32464; TRIP2 in figures). From the Vienna *Drosophila* Resource Center (VDRC): *Egr-GFP* (VDRC 318615), *UAS-dTRAF1-RNAi* (VDRC 110766), *UAS-grnd-RNAi* (CG10176, VDRC 104538; VDRC1 in figures), *UAS-grnd-RNAi* (CG10176, VDRC43454; VDRC2 in figures), *UAS-wgn-RNAi* (GD9152, VDRC 9152), *UAS-dTRAF2-RNAi* (VDRC 110266), *UAS-p38-RNAi* (CG7393, VDRC 330146), *UAS-Tak1-RNAi* (CG18492; VDRC330457).

Immunolocalization

The primary antibodies used in this work were against phospho-p38 from rabbit (1:50, (Cell Signaling Technology Cat# 9211, RRID:AB_331641)), rabbit anti-cleaved Death Caspase-1 (Dcp1) (1:200, (Cell Signaling Technology Cat# 9578, RRID:AB_2721060)), β -galactosidase (mouse 1:1000, (Promega Cat# Z3783, RRID:AB_430878)), Wengen (mouse 1:100, gift from K. Basler), Grindelwald (guinea pig 1:500, gift from Pierre Leopold). The fluorescently labeled secondary antibodies were from Life Technologies, all used 1:200 in 0.3% Triton-PBS: goat anti-mouse Alexa Fluor 488 (Thermo Fisher Scientific Cat# A-11001, RRID:AB_2534069), donkey anti-rabbit Alexa Fluor 568 (Thermo Fisher Scientific Cat# A10042, RRID:AB_2534017), and goat anti-guinea pig Alexa Fluor 555 (Thermo Fisher Scientific Cat# A-21435, RRID:AB_2535856).

Wing imaginal discs dissected from late third instar larvae in 1×PBS (pH 7.4) were fixed in 4% paraformaldehyde (Electron Microscopy Sciences, Cat# 15710) in PBS for 40 minutes at room temperature, washed in PBS 0.3% Triton X-100 (PBT), blocked for 2 hours

in PBT containing 2% BSA, and incubated overnight with primary antibodies at 4 °C. The next day, the discs were washed and incubated with secondary antibodies for 2 hours at room temperature. After washing, nucleic acid staining was performed by incubating the discs for 10-15 minutes with the nuclear marker TO-PRO-3, 1mM (TP-3, Thermo Fisher Scientific Cat. # T3605) or DAPI, 10 mM (Thermo Fisher Scientific, Cat.# D21490). The discs were mounted in SlowFade Diamond Antifade Mountant (Thermo Fisher Scientific, Cat.# S36967).

Image acquisition

For the confocal images, a Zeiss LSM880 and a Leica SPE confocal laser scanning microscopes were used. Images were analyzed and processed using FIJI. A Leica DMBL microscope was used for taking pictures of the adult wings.

Quantification of the Wgn and Grnd response to damage

Stacks of images corresponding to Wgn and Grnd localization in these three genotypes were used for quantification: (1) *sal^{E/Pv}>rpr*; (2) *sal^{E/Pv}>rpr, Sod1:Cat*; and (3) *sal^{E/Pv}>rpr, egr RNAi*. Mean pixel intensities were collected from raw images taken with the same confocal settings. Rectangular ROIs were traced to measure mean pixel intensity in two regions of the same disc (A: anterior, P: posterior), both outside the death zone.

Statistical analysis and cell death area ratio calculation

To calculate the cell death area ratio, we used FIJI. First, we generated a Z-projection of the whole stack for the anti-Dcp1 channel using the Max intensity projection. Then, we applied the remove outliers tool (radius 2, threshold 10). Subsequently we thresholded the image with the MaxEntropy Threshold to create a binary image and automatically determine the area in pixels of the dead zone with the analyze particles tool. The data was normalized dividing the area of Dcp1-positive cells by the total area of the imaginal disc. As a result, the cell death area ratio was obtained.

In Figures 1 and 3, the data are in mean $\pm$ SD. To make statistical comparisons, we used a one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test to make pair comparisons between each group using GraphPad. Significance is indicated in the figures only when $p < 0.05$, as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

In figure S6B we used a two-tailed Student's t-test to make the statistical comparisons using GraphPad (**** $p < 0.0001$).

Gal4/UAS/Gal80ts for *eiger^{weak}* activation in the wing imaginal disc

The Gal4/UAS transactivation system was temporarily controlled by the *tub-Gal80^{TS}* thermo-sensitive Gal4-repressor. *Egr/TNF α* was ectopically expressed using the *UAS-eiger^{weak}* construct (Moreno, Yan, et al., 2002), which results in reduced *egr* activity and therefore low levels of apoptosis. The expression of the transgene was controlled by the thermo-sensitive Gal4 repressor *tub-Gal80^{TS}*.

Drosophila of the desired genotype were cultured to lay eggs for 24 hours at 17 °C. Conditions for experiments in Figures 1 and 3: Embryos were kept at 17 °C until the 8th day (192 hours) after egg laying to prevent *UAS-eiger^{weak}* expression. The larvae were subsequently transferred to 29 °C for 16 hours and then the imaginal discs from wandering larvae were dissected and processed for staining and immunofluorescence studies.

Conditions for experiments in Figure 4: To enhance the production of *egr*-dependent apoptotic cells, embryos were kept at 17 °C until the 7th day (168 hours) after egg laying to prevent *UAS-eiger^{weak}* expression. The larvae were subsequently transferred to 29 °C for 24

hours and then the imaginal discs from wandering larvae were dissected and processed for staining and immunofluorescence studies.

ROS detection *ex vivo*

All experiments for ROS detection were done in living conditions. To detect the presence of ROS, we used the MitoSOX reagent (Thermo Fisher Scientific, Cat. #M36008), which is an indicator of oxidative stress of mitochondrial origin in living cells. Third instar discs were dissected in Schneider's medium immediately after cell death or injury and incubated in agitation for 15 minutes in medium containing 5 μ M MitoSOX reagent, followed by three washes in Schneider's culture medium (Sigma-Aldrich S0146). The samples were protected from light throughout the experiment. They were then mounted using culture medium supplemented with 1 μ M TO-PRO-3 for nucleic acid staining. As *in vivo* TO-PRO-3 only enters dying and dead cells, we used it to distinguish dead cells from living cells.

Genetic ablation and the dual Gal4/LexA transactivation system

For adult wing regeneration analysis, we used a dual Gal4 and *lexA* transactivation system, as described previously (Esteban-Collado et al., 2021; Santab rbara-Ruiz et al., 2019). Briefly, the *lexA/lexO* system was used for genetic ablation after induction of apoptosis, and the Gal4/UAS system for activating the desired transgene. The *UAS-wgn-RNAi* and *UAS-grnd-RNAi* transgenes were activated under the control of *ci-Gal4*, *ap-Gal4* or *nub-Gal4* (Fig. 6 and Fig. EV6).

The genetic ablation system used to study regeneration was *sal^{E/Pv}-LHG*, *tub-Gal80^{TS}*, *lexO-rpr*. Apoptosis was induced during larval stages in the wing-specific *sal^{E/Pv}* domain using the *Gal80*-repressible transactivator system LHG (LexA-Hinge-Gal4 activation domain), a modified form of the *lexA/lexO* system (*-LHG>lexO-rpr*) (Yagi et al., 2010) (Fig. 6A).

Flies of the desired genotype were allowed to lay eggs during 6 hours at 17  C. Eggs were allowed to develop on standard fly food. Genetic ablation was achieved after temperature shifts from 17  C to 29  C of 11 hours in vials containing the synchronized larvae.

The ability of the wing disc to regenerate after genetic ablation has been associated with the induction of a developmental delay (Colombani et al., 2012; Garelli et al., 2012; Jaszczak et al., 2015; Katsuyama et al., 2015; Smith-Bolton et al., 2009). All genotypes analyzed in Figure 6 showed a similar developmental delay of 1-2 days (at 17  C) after genetic ablation in comparison with the animals of the same genotype in which no genetic ablation was induced, i.e. developed continuously at 17  C (Fig. EV6A). After the adults emerged, the wings were dissected, and regeneration was analyzed.

To test the capacity to regenerate, we used adult wings from *sal^{E/Pv}>rpr* individuals, in which patterning defects can be easily scored. Flies were fixed in glycerol:ethanol (1:2) for 24 hours and the wings were dissected in water and then washed with ethanol. They were then mounted in 6:5 lactic acid:ethanol and analyzed and imaged under a Leica microscope.

The areas of the mounted wings were outlined and scored using FIJI. In addition, we divided the wings into three categories depending on the severity of the defects in terms of pattern and number of absent veins and interveins: regenerated (normal pattern), mild aberrant phenotype (1-2 veins missing), and strong aberrant phenotype (>3 veins missing or more aberrant phenotype).

The use of the knock down RNAi transgenes alone did not affect vein pattern or wing size (Fig. EV6A). Also, flies carrying the constructs shown in Figure 6 but raised constantly at 17  C to maintain *tub-Gal80^{TS}* activity and block transgene (*UAS* or *lexO*) expression did not show defects in wing size or pattern (Fig. EV6A).

This double transactivation system was also used to dissect imaginal discs after genetic ablation and *grnd* or *wgn* downregulation and score the effects on phospho-p38 localization (Fig. 6). In these experiments, we used the same conditions as those for adult wings and the *nub-Gal4* driver was selected because its expression domain surrounds the *sal*^{E/Pv} zone.

Characterization of Grnd and Wgn gene family evolution

Protein sequences of TNFRs were researched using the blastp and tblastn software from the online Blast server (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>), restricting by taxonomy to specifically search in the genomes and proteomes of those animal lineages of particular relevance for Grnd and Wgn evolution. *Drosophila melanogaster* Grnd and Wgn were used as initial queries. After that initial search, a second one was performed using the retrieved protein hits as new queries. The same procedure was used in two slow-evolving deuterostome lineages (a cephalochordate, the European amphioxus *Branchiostoma lanceolatum*, and a hemichordate, the acorn worm *Saccoglossus kowalevskii*), using *Homo sapiens* TNFRs as initial queries.

For the phylogenetic analyses of TNFRs, protein sequences were aligned with the MAFFT software (Katoh et al., 2019) and the resulting alignments were trimmed using Aliview to discard spuriously aligned regions (Larsson, 2014). Phylogenetic trees were built using IQ-Tree (Nguyen et al., 2015), testing the tree with UFBoot (bootstrap = 103) (Hoang et al., 2018) and an approximate Bayes test for single branch testing. The model used was selected using ModelFinder with BIC as the criteria (Kalyaanamoorthy et al., 2017). The trees were visualized using ITOL (Letunic & Bork, 2021).

Two phylogenetic trees were built. The first one was done using only the CRDs from all the arthropod and deuterostome TNFRs retrieved in our searches, except for a few highly divergent sequences from Ostracoda and Thecostraca (1 from *Cyprideis torosa* and 2 from *Amphibalanus amphitrite*). In this tree, all arthropod TNFRs were assigned with high support to either Grnd or Wgn monophyletic groups, except in the case of a TNFR from the spider *Argiope bruennichi*, which grouped together with some deuterostome sequences with very low support (Fig. 2A and EV2A). The second tree was built using the full protein sequences of Wgn and Grnd from different arthropods, together with the few remaining arthropod TNFR sequences that could not be classified in the previous tree (the 4 sequences from *C. torosa*, *A. amphitrite* and *A. bruennichi*), which in this case were successfully assigned as Grnd or Wgn orthologs (Fig. EV2B).

For the CRD tree, the corresponding protein domains were extracted using Hmmer 3.4 (<http://hmmer.org/>). LIM domains, which are also cysteine-rich domains unrelated to those of TNFRs, from the human proteins ISL1, LIMCH1, and FHL1 were used as an outgroup. The model selected for this tree was PMB+G4.

The Grnd and Wgn tree was built with the model VT+I+G4+F. Three TNFRs from deuterostomes were used as outgroups, TNFR6L from *B. lanceolatum* (CAH1240663.1), TNFR11L from *S. kowalevskii* (XP_006819214.1), and TNFR1B from *H. sapiens*.

A PCA-based alignment of all the CRDs used in the previous phylogenetic analyses was built using the Jalview software (Waterhouse et al., 2009) and represented with R. Also, an all versus all comparison was performed using blastp. The percent identity was normalized with the total length of the query. The results were represented using R (github.com/rlbarter/superheat).

Figure legends

Figure 1. Induction of apoptosis in Egr/TNF α cells after downregulation of different TNFR pathway genes.

(A-H) Dcp1-positive cells (red) after ectopic co-expression of *egr^{weak}* and (A) GFP-expressing control (n=12), (B) *grnd RNAi* (n=15), (C) *dTRAF2 RNAi* (n=18), (D) *Tak1 RNAi* (n=9), (E) *wgn RNAi* (n=18), (F) *dTRAF1 RNAi* (n=10), (G) *Ask1 RNAi* (n=8) and (H) double RNAi of *wgn* and *grnd* (n=12). TP3 was used to stain nuclei. Scale bar: 50 μ m. (I) Cell death area ratio calculated in base 1 for the genotypes indicated. Box plots show maximum-minimum range (whiskers), upper and lower quartiles (open rectangles), and median value (horizontal black line), each dot representing the cell death area ratio from a different imaginal disc. Gray dots: a disc of each genotype is shown in (A-H); Black dots: a disc of each genotype in EV1). n.s.=not significant, ** $p=0.0002$, **** $p<0.0001$.

Figure 2. Evolution of Wgn and Grnd.

(A) ML phylogenetic tree of the CRDs of Wgn, Grnd, and deuterostome TNFRs. *Drosophila melanogaster* Wgn and Grnd are indicated by solid green and light blue circles, and their corresponding monophyletic clades are colored accordingly. The outgroup branch, containing the cysteine-rich sequences of LIM proteins, is indicated in gray. (B) Wgn and Grnd presence/absence in different arthropod lineages. The presence of the two gene families is indicated by solid squares; white squares indicate putative gene losses. Divergence times of crown arthropods and crown pancrustaceans are indicated at the corresponding nodes of the arthropod tree. (C) PCA-based alignment of the CRDs of Wgn, Grnd, and Deuterostome TNFRs.

Figure 3. The p38 MAP2 kinase *lic* rescues the *wgn* mutant phenotype.

(A, B, C) Dcp1-positive cells after *egr^{weak}* overexpression and *wgn RNAi* in (A) *wgn RNAi* (n=10), (B) *wgn RNAi* and *lic^{WT}* (n=8), (C) *wgn RNAi* and *hep^{WT}* (n=10). (D) Cell death area ratio calculated in base 1 for the genotypes indicated. Box plots show maximum-minimum range (whiskers), upper and lower quartiles (open rectangles), and median value (horizontal black line). * $p=0.0314$, *** $p=0.0002$. TP3 was used to stain nuclei. Scale bar: 50 μ m.

Figure 4. Wgn accumulation after ectopic *egr/TNF α* expression is abrogated after enzymatic depletion of ROS.

All transgenes were ectopically expressed in the posterior compartment (*hh>*). The wing pouch of each genotype (above) is complemented with an orthogonal apico-basal section (below); white lines indicate the level of the z-axis of the above images. (A) Anti-Wgn and Dcp1 staining of control wing discs (n=11). (B) Anti-Grnd and Dcp1 staining of control wing discs (n=4). (C) Anti-Wgn and Dcp1 staining of *egr^{weak}* (n=10). (D) Anti-Grnd and Dcp1 staining of *egr^{weak}* (n=7). E, F, G Discs stained ex-vivo after *egr^{weak}* activation. Left: cell death (TP3). Right: ROS of mitochondrial origin (MitoSOX) in (E) *wgn RNAi*, (F) *wgn RNAi* and ectopic expression of p35, (G) *wgn RNAi* and ectopic expression of *Sod1:Cat*; dotted lines indicate the contour of the imaginal discs. (H) Anti-Wgn and Dcp1 staining of *egr^{weak}* and *Sod1:Cat* (n=8). (I) Anti-Grnd and Dcp1 staining of *egr^{weak}* and *Sod1:Cat* (n=8). Scale bars: 50 μ m.

Figure 5. Wgn accumulation after ectopic activation of the pro-apoptotic gene *rpr* is abrogated by enzymatic depletion of ROS.

The wing pouch of each genotype (above) is complemented with an orthogonal apico-basal section (below); white lines indicate the level of the z-axis of the above images. (A) Anti-Wgn and Dcp1 of disc with genetically induced apoptosis using $sal^{E/Pv}>rpr$ (n=12). (B) Anti-Grnd and Dcp1 of disc with genetically induced apoptosis using $sal^{E/Pv}>rpr$ (n=10). (C) Anti-Wgn and Dcp1 of $sal^{E/Pv}>rpr$, *Sod1:Cat* (n=16). (D) Anti-Grnd and Dcp1 of $sal^{E/Pv}>rpr$, *Sod1:Cat* (n=8). (E) Anti-Wgn and Dcp1 of $sal^{E/Pv}>rpr$, *egr RNAi* (n=19). (F) Anti-Grnd and Dcp1 of $sal^{E/Pv}>rpr$, *egr RNAi* (n=10). Scale bars: 50µm. (G, H) Box plots of the mean pixel intensities of $sal^{E/Pv}>rpr$, $sal^{E/Pv}>rpr$, *Sod1:Cat* and $sal^{E/Pv}>rpr$, *egr RNAi* after Wgn (G) and Grnd (H) immunostaining. ** $p=0.0015$ and **** $p<0.0001$, n.s.=not significant. Quantification was done in the anterior (A) and posterior (P) compartment of each imaginal disc (rectangles in the imaginal disc representation).

Figure 6. Knockdown of *wgn* but not *grnd* impairs p38-dependent regeneration.

(A) Design for ectopic expression of transgenes and simultaneous apoptosis induction. Purple area in disc: apoptosis induced by $sal^{E/Pv}$ -*LHG, LexO-rpr*. Light purple in adult wing: regenerated tissue. The *Gal4/UAS* activates RNAi in *ci*/anterior compartment (yellow), *ap*/dorsal compartment (blue). Adult wings were scored for complete regeneration of the missing zone. $sal^{E/Pv}$ -*LHG* and *Gal4* are under the control of *tub-Gal80^{TS}*. Apoptosis was induced by shifting the temperature to 29 °C for 11 hours. The larvae were transferred to 17 °C, where they regenerated and emerged into adults, in which wings were scored. (B) Regeneration assay. Box plot: Y-axis shows the average area in pixels of adult wings obtained after apoptosis in the $sal^{E/Pv}$ region and the RNAi or mutant (genotype, X-axis). Each dot represents one wing; wild-type pattern/regenerated (gray), mild aberrant phenotype (yellow) and strong aberrant phenotype (purple), n.s.= not significant, **** $p<0.0001$. (C-F) Activated p38 in regeneration assay of wing discs. Phosphorylated p38 (P-p38) in non-ablated disc (C; n=10), in $sal^{E/Pv}$ -*LHG, LexO-rpr* apoptosis and *nub-Gal* activation of *GFP* (D; (n=8)), *UAS-grnd-RNAi* (E; (n=18)), and *UAS-wgn-RNAi* (F; (n=12)). White lines in the confocal images outline the $sal^{E/Pv}$ -*LHG, LexO-rpr* dark area full of pyknotic nuclei of apoptotic cells. TP3 was used to stain the nuclei. Scale bar: 50µm.

Figure 7. Model for ROS-dependent Wgn recruitment for survival and regeneration.

ROS are required for the oxidation of thioredoxin (Trx) to dissociate from Ask1. ROS are required for the phosphorylation of Akt, which in turn phosphorylates Ask1 to divert its function towards survival and regeneration. ROS are required for Wgn recruitment in the Ask1/p38 axis. Phosphorylation of Ask1 by Akt results in tolerable levels of p38 and JNK (perhaps very low, gray) activity and the avoidance of apoptosis. In contrast to Wgn, Egr-Grnd interaction triggers JNK-dependent apoptosis.

Figure Expanded View 1. Dcp1-positive cells in different RNAi strains expressed under *hh-Gal4*.

(A) Control *egr^{weak}* overexpression only. (B) *egr^{weak}* and *wgn RNAi* from VDRC. (C) *egr^{weak}* overexpression and *wgn RNAi*, TRiP strain. (D) Control expression of *wgn RNAi*, VDRC alone. (E) Control expression of *wgn RNAi*, VDRC alone. (F) *egr^{weak}* and *grnd RNAi*, TRiP. (G) *egr^{weak}* and *sTRAF2 RNAi*, TRiP. (H) *egr^{weak}* and *Tak1 RNAi*, VDRC. (I) *egr^{weak}* and *dTRAF1 RNAi*, TRiP. (J) *egr^{weak}* and *Ask1 RNAi*, VDRC. TP3 was used to stain nuclei. Scale bar: 50µm.

Figure Expanded View 2. (A) Full version, including all the species names and sequence accession numbers of the ML phylogenetic tree of the CRDs of Wgn, Grnd, and deuterostome

TNFRs featured in Fig. 2A. *B. lanceolatum*, *S. kowalevskii*, and *H. sapiens* are abbreviated as Bla, Sko and Hsa, respectively. (B) ML phylogenetic tree of all identified arthropod proteins, using full-length protein sequences and three deuterostome TNFRs as outgroups, *B. lanceolatum* TNFR6L (CAH1240663.1), *S. kowalevskii* TNFR11L (XP_006819214.1), and *H. sapiens* TNFR1B. (C) Pairwise comparison of Wgn, Grnd, and deuterostome CRDs. (D) CRD alignments of some representative Wgn and Grnd proteins.

Figure Expanded View 3. Reduction or increase of p38 activity results in an increase of apoptosis in *egr^{weak}* tissue. (A) *UAS-p38-RNAi* and *UAS egr^{weak}* transgenes were co-expressed under *hh-Gal4*. (B) *UAS-lic^{WT1.1}* and *UAS egr^{weak}* transgenes were co-expressed under *hh-Gal4*. Nuclei staining was done with TO-PRO-3 and apoptosis with the caspase Dcp-1. Scale bar: 50µm.

Figure Expanded View 4. (A, B) *Hh-Gal4 UAS-egr^{weak}* wing disc to show pyknotic nuclei positive for the cleaved caspase Dcp1 in apical-basal polarity. (A) Plane close to the apical side of the epithelium with a high magnification of the square zone. Note that pyknotic nuclei coincide with Dcp1 cells (e.g. arrows). (B) Plane close to the basal side of the epithelium with a high magnification of the square zone. Note that pyknotic nuclei coincide with Dcp1 cells (e.g. bracket), and that the concentration of apoptotic cells is more abundant in the basal area. (C) Discs stained *ex-vivo* after co-expression of *egr^{weak}* and *wgn RNAi*. Cell death (TP3), ROS of mitochondrial origin (MitoSOX). (D-F) Phosphorylation of p38 after ectopic expression of *egr^{weak}*. (D) The transgene *egr^{weak}* was activated in the posterior compartment (*hh-Gal4*); in this optical section there is an accumulation of pyknotic nuclei, typical of apoptotic cells in the center of the posterior wing pouch. Phospho-p38 is found in cells surrounding the apoptotic cells. (E) The expression of the apoptosis inhibitor *p35*, and (F) the ROS scavengers *Sod1::Cat* concomitantly with *egr^{weak}* result in a strong reduction of phospho-p38. Scale bar: 50µm.

Figure Expanded View 5. (A-C) High magnification of the interphase between dying cells and responding tissue corresponding to Figure 5. (A) Anti-Wgn and Dcp1 of disc with genetically induced apoptosis using *sal^{E/Pv}>rpr*. (B) Anti-Wgn and Dcp1 of *sal^{E/Pv}>rpr, Sod1::Cat*. (E) Anti-Wgn and Dcp1 of *sal^{E/Pv}>rpr, egrRNAi* (n=19). (D-E) Apoptosis genetically induced in *ptc>rpr* in two different Egr reporter backgrounds; (D) *Egr2xGFP* reporter and (E) *EgrLacZ* detected by anti-β-Gal antibody. The yellow zone in the merged image shows colocalization of β-Gal-positive cells and Dcp1-positive cells (dead cells). The dotted lines outline pyknotic nuclei of apoptotic cells. TP3 was used to stain the nuclei. Scale bar: 50µm.

Figure Expanded View 6. Control for regeneration assay. (A) Box plot: Y-axis shows the average area in pixels of adult wings obtained from controls kept at 17 °C, with no cell death induction (*sal^{E/Pv}-LHG, LexO-rpr* OFF) and no expression of the transgenes. It also shows the average area in pixels from adult wings after the sole expression of the RNAi or mutant background (genotypes indicated in the X-axis). Each dot represents one wing: wild-type pattern (gray). (B) Regeneration assay. Box plot: Y-axis shows the average area in pixels of adult wings obtained after apoptosis in the *sal^{E/Pv}* region and the *wgn RNAi* in the *nub* zone (as in Fig 6F, G). Each dot represents one wing; wild-type pattern/regenerated (gray), mild aberrant phenotype (yellow) and strong aberrant phenotype (purple), *****p*<0.0001. (C) Regeneration assay. Box plot: Y-axis shows the average area in pixels of adult wings obtained after apoptosis in *sal^{E/Pv}>rpr*, in *sal^{E/Pv}> wgn RNAi* and in *sal^{E/Pv}>rpr + wgn RNAi*. Each dot represents one

wing; wild-type pattern/regenerated (gray), mild aberrant phenotype (yellow), and strong aberrant phenotype (purple), n.s.= not significant.

Supplementary table 1. Wgn and Grnd gene complements in arthropods.

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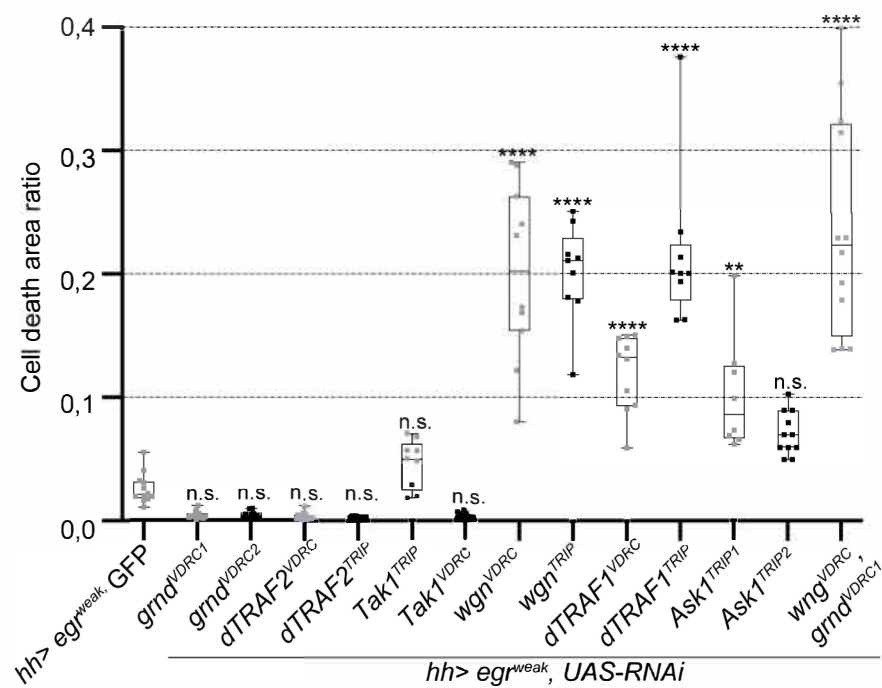
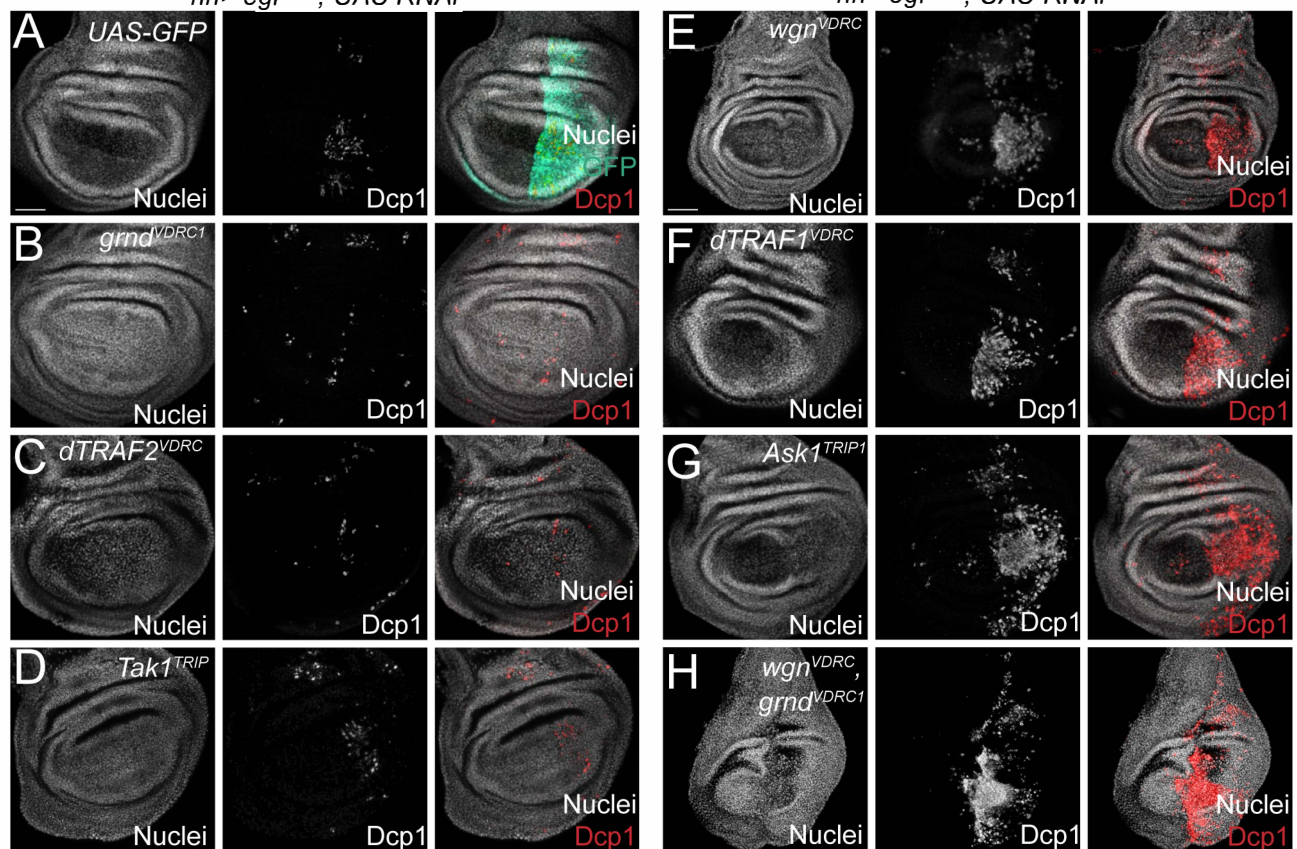


Figure 1

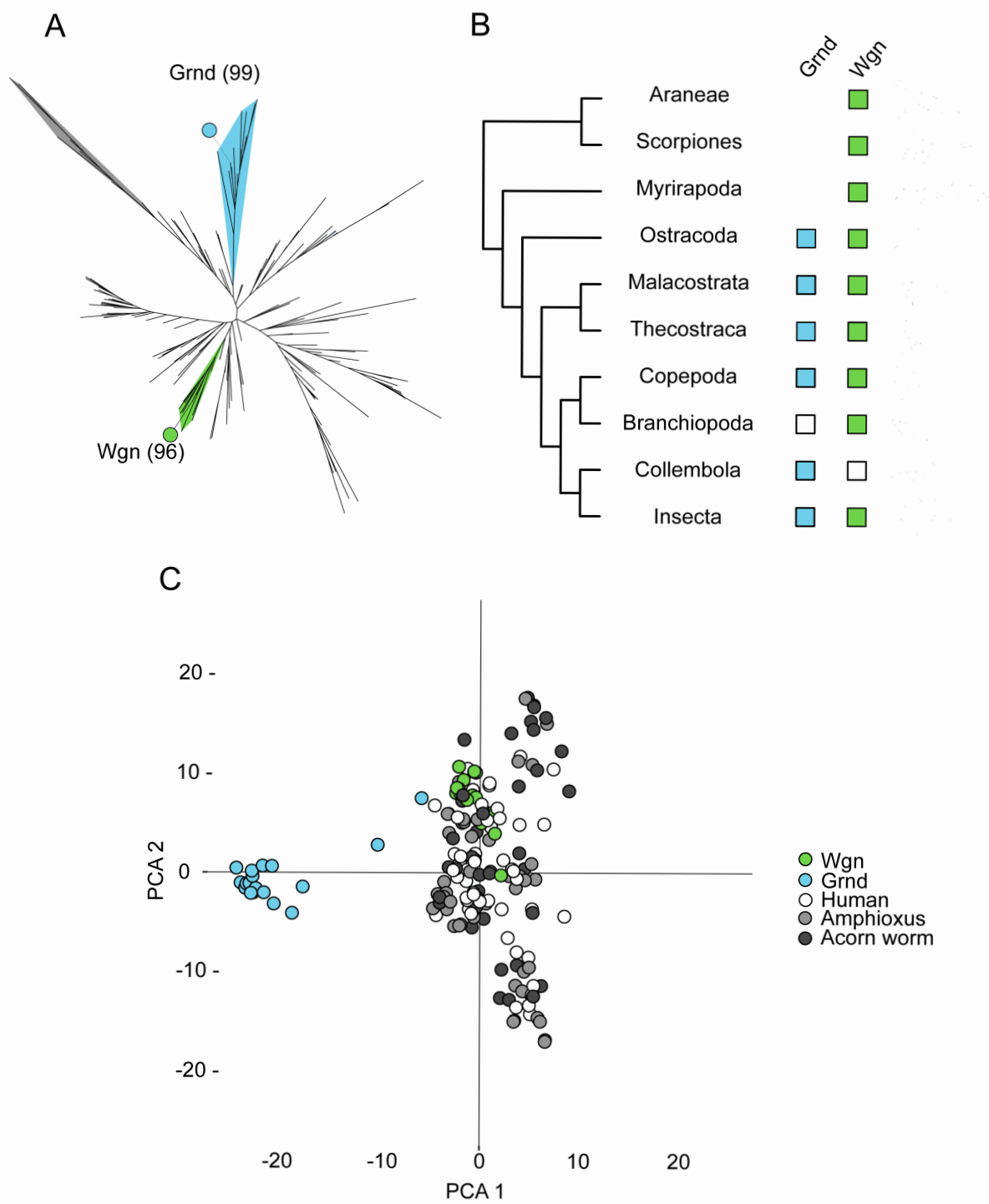
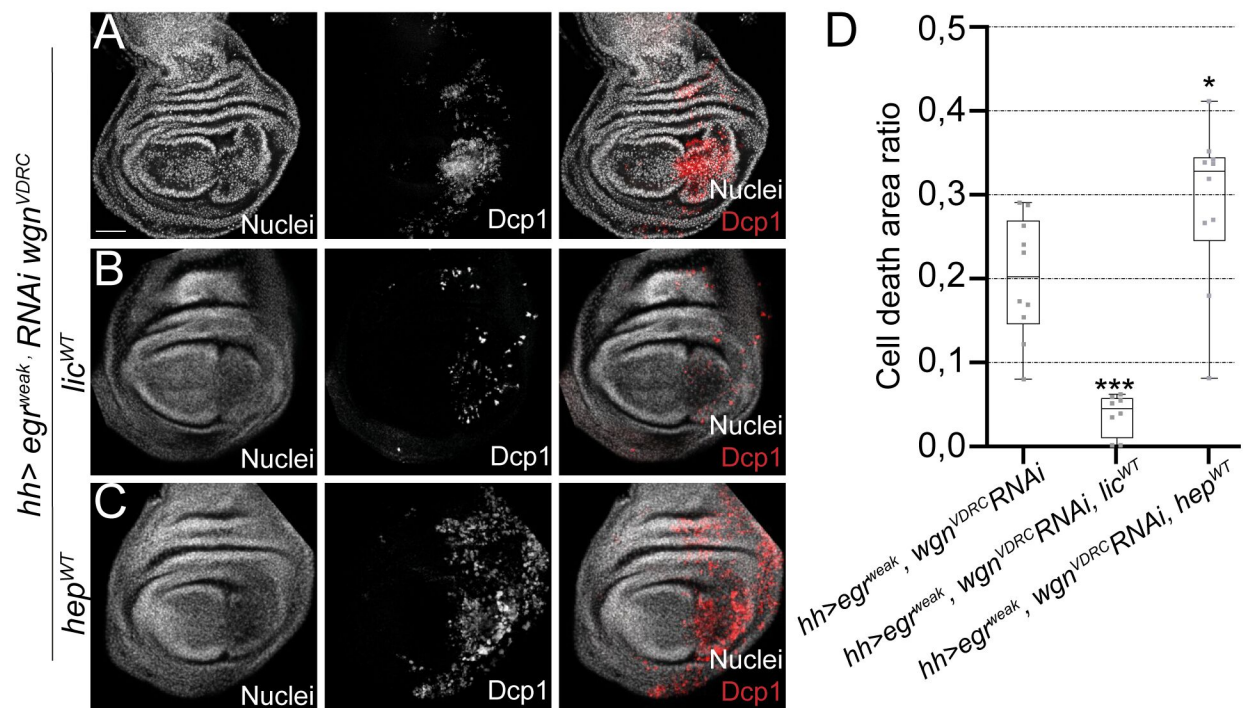
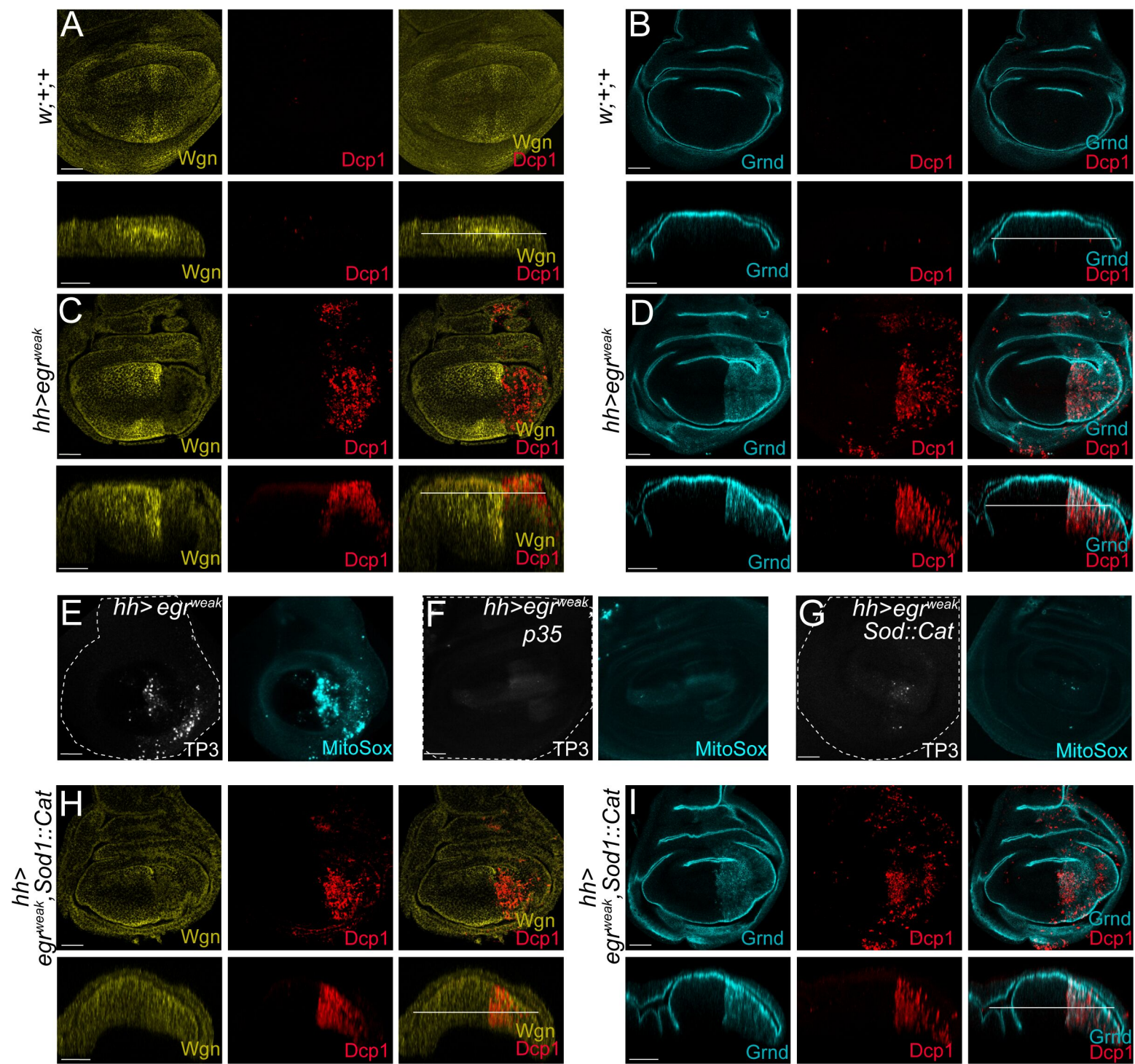
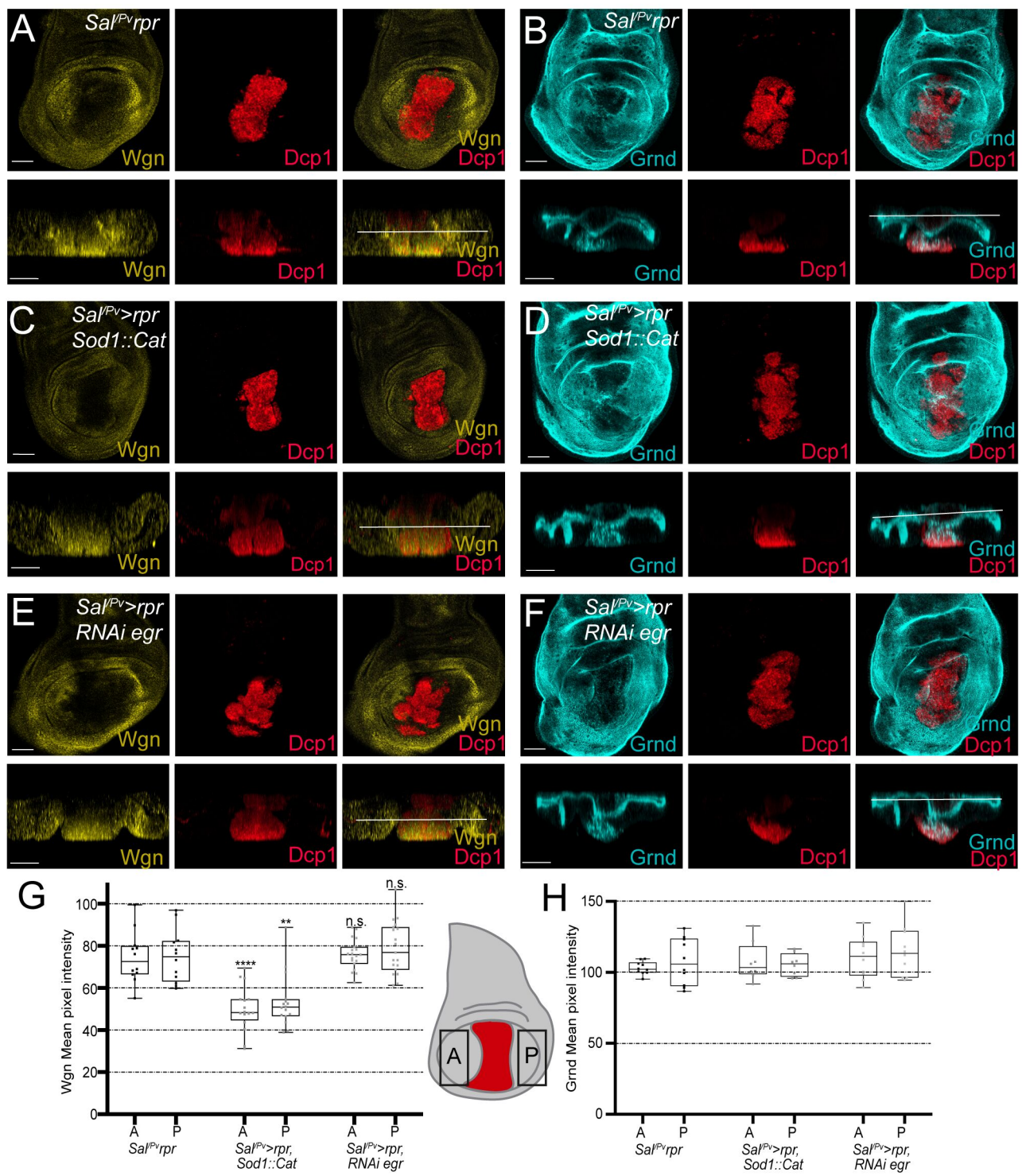
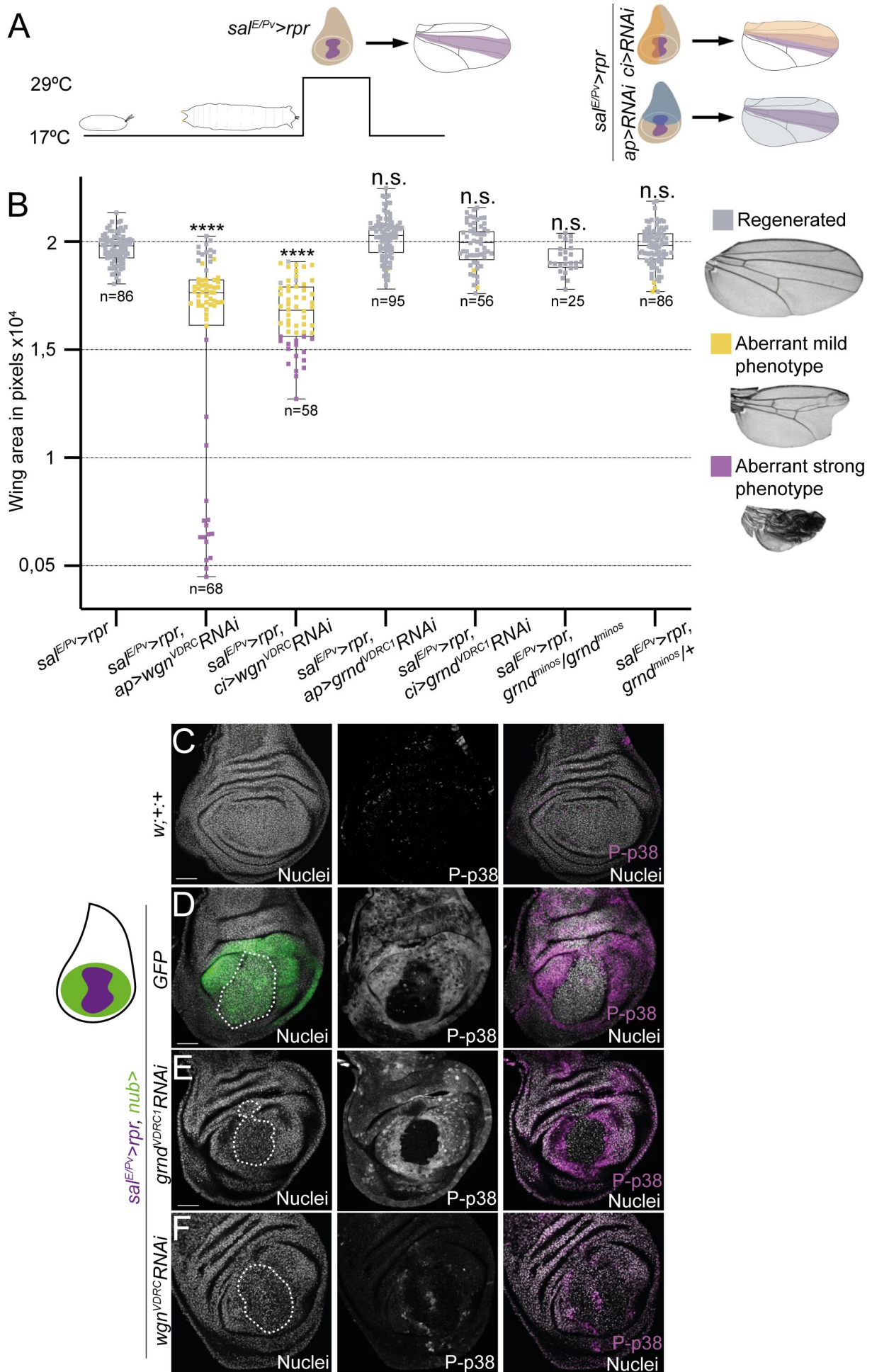


Figure 2









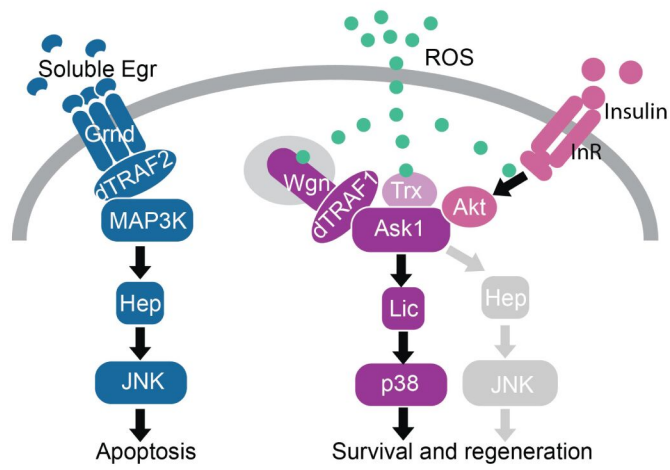
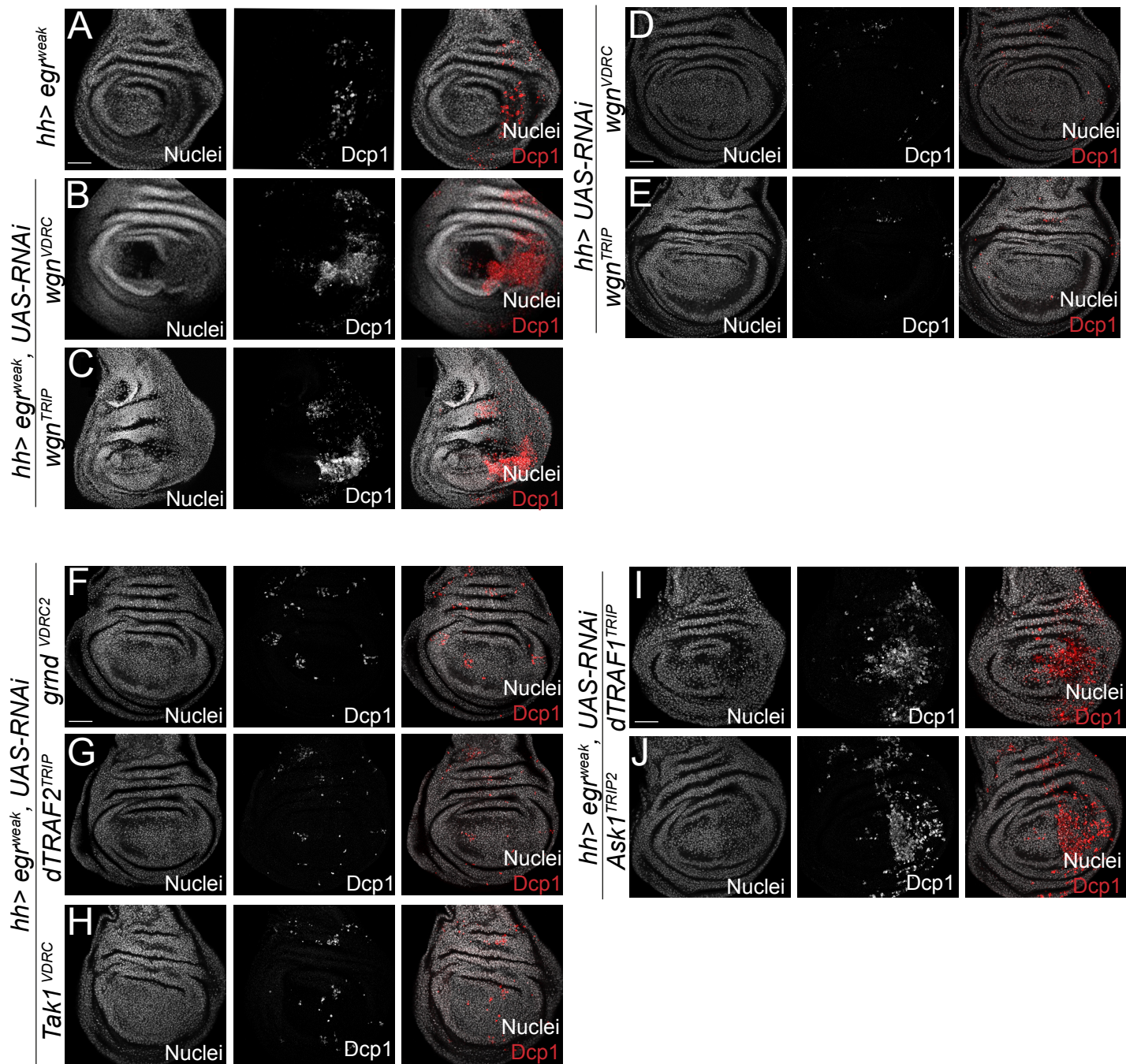


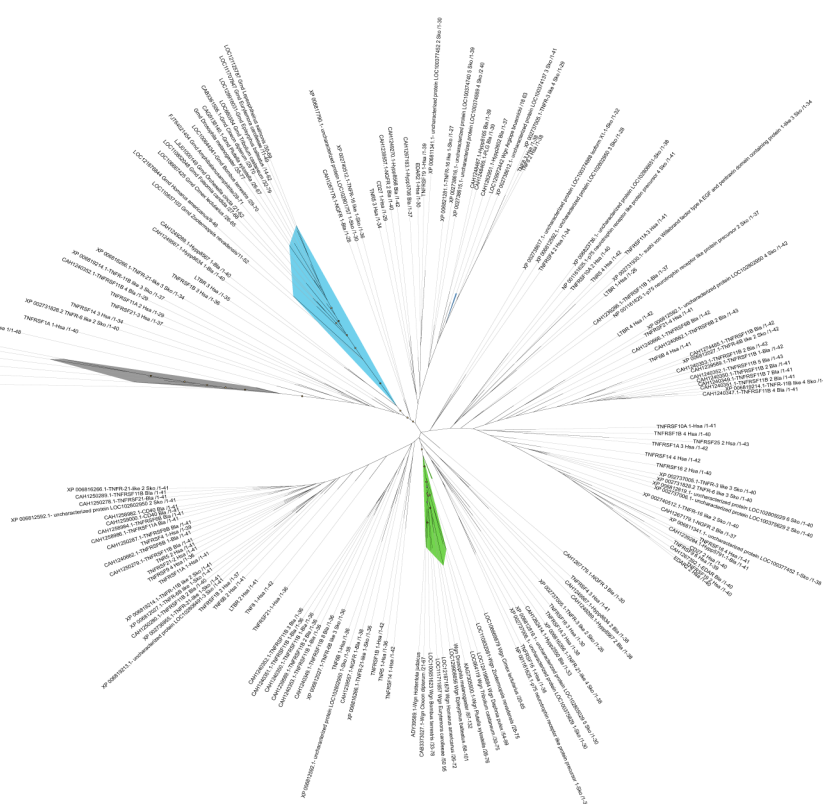
Figure 7



A

Branch support

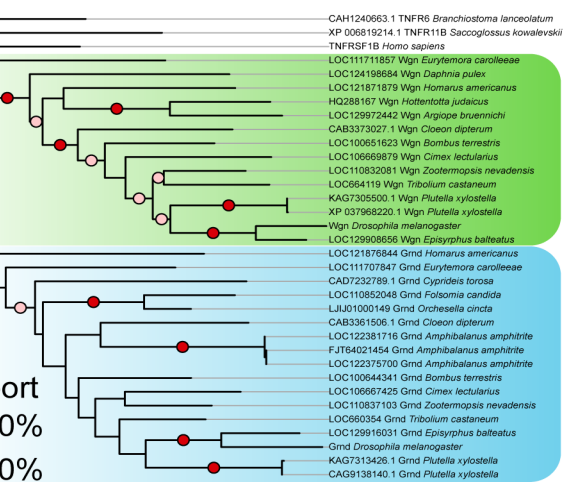
- > 90%
- > 80%
- < 80%



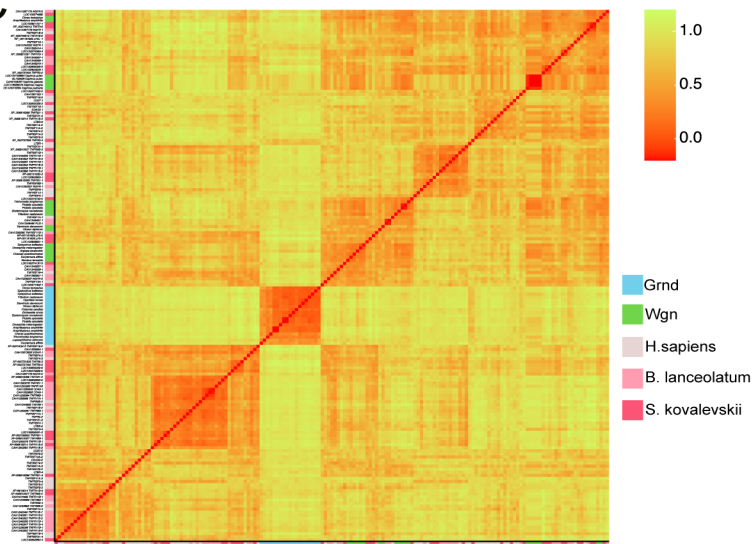
B

Branch support

- > 90%
- > 70%



C



D

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Wgn-[Drosophila melanogaster]/87-132
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LOC664119-Wgn-[Tribolium castaneum]/30-75
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LOC110832081-Wgn-[Zootermopsis nevadensis]/28-75
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LR903237-Wgn-[Darwinula stevensoni]/52-96
LOC129972442-Wgn-[Argiope bruennichi]/18-63

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10 20 30 40 50
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SPC - APQHWWD SQDR - - CTPOTRC - QGEMI - PLRPCQLHRTDI CGSIYDL
AVCERGRSHWDRRAGA - - CAPCRLCDPARRLAVLLPCELHRDITVCHALQHI
SLC - VSRQYFNRLAS - - CVMOTEC - VEGDI - VVRPCFHRDITLCRPIKEL
PVCKPGEFWSVERAT - - CWPOTRC - APEF - TLSPCAVYKDAICGPLSAL
ALCRPHGQFWD ET SER - - CVMOTEC - VLRPCQVHODTQCGPLSAL
RLC - NLNEYWSNEEEI - - CLPOTKC - PPHQL - TMRPCQGHKDTVCCKPMSNL
PPCPKGHGFWDTNRQE - - CAQKVC - SHNEV - VLMPCPQYKDTLCGPMSAL
DFCEQWVAFKDPQTGR - - CLPOTKC - PEDHL - TVAKCEFDRTDITLCRPLTDL
- - CRRVFF - FWDTGKDK - - CLPOTRC - QTRGLV - TLLPCQYFRDSVCVKSSDW
LSCQGGQEFYNSEQGR - - CVPOTRC - HGSLV - VVPCYVYRDVAVCAPAAQF
HACIEGTYSD - - TSEILD - CFLSGC - GDGLF - ELHACNPFRTDITCDS - -
ELQKQKEWEND - MTGK - - COPOTVC - AGKSW - EMAPCLDFSITLCVNLI E

10 20 30 40 50
CNN - AKVCSL - EEEYCTFSERCEKCEEICTISHNFHESDNKYCPAYGND
CHG - TICHVP - NEFYVATERCHPCEI EVNNQTHNYDAFLCAKESAYKTF
KCG - QLKCAA - DDYCSPTDRACAPCSDVCTKTGHNFDEGLCKEQQYIHN
SGC - QKKCKR - DEYCTSYTKACEPCKSKICKSTHNFDETECVKKQDYIHD
DGC - EQRCS - VEYCSYDKRCKPSSICDATTGRNYQQNEICKDEYELHD
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PCG - EKSCSR - YEYCSATDY - - QNCEPCTVGERNFDSVICKEQEYIHD
LCG - VHTCSD - NOYCSNFGNICSDCSNICDLKSHNYEHQECFCKQGYIHD
- CG - EIVCKA - HYYCARIDDSRCPQETCDRALSNYDETECKDQDYLDH
- CG - DIICQP - HNYCSQIDHTCRPQVACDPTLGNYDKGVCEKCDQDLHD
TCS - SDLPCTDLYSYCDTFLSRQPCSQLC - - KDNFSE - - CHQCEGYLQS
RCG - TDHTCSL - HQYCSPATGTCEACDYICHPGTKNYGRDRCECTSCQDYLDH
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Figure supplementary 2

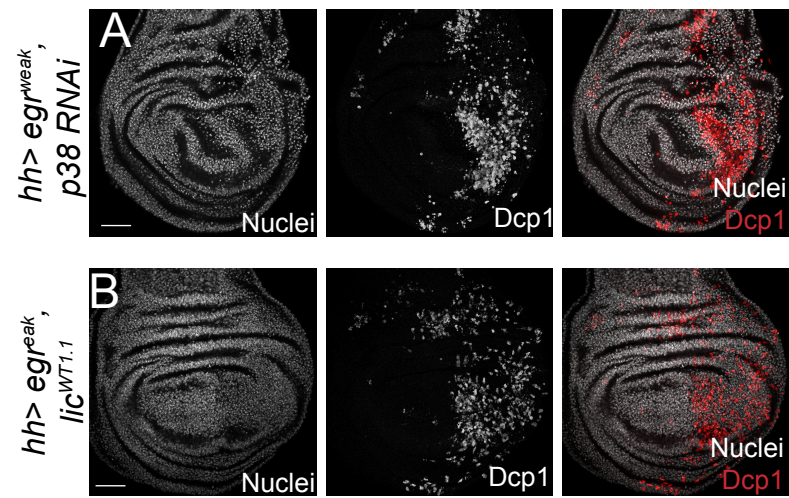


Figure Expanded View 3

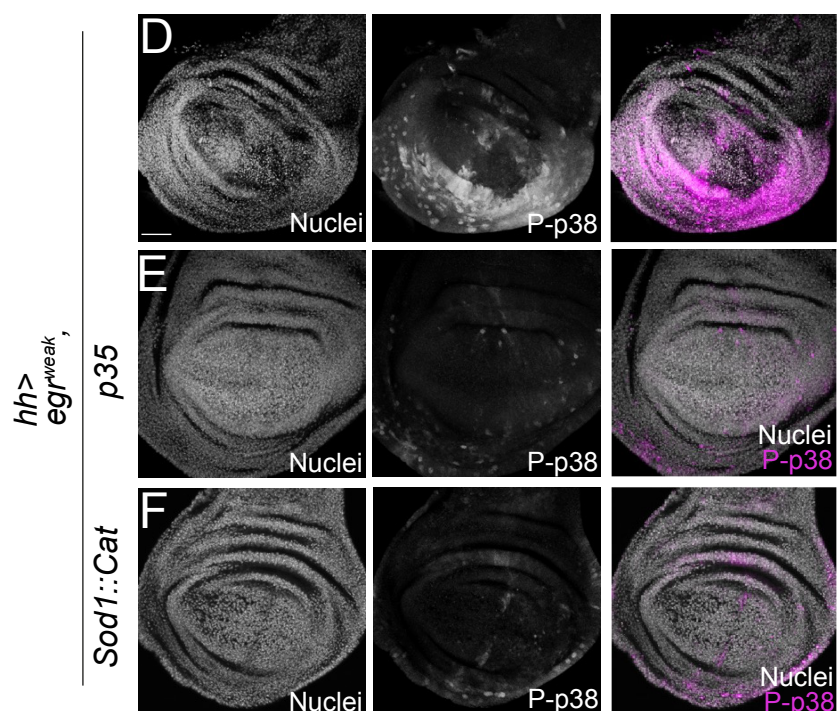
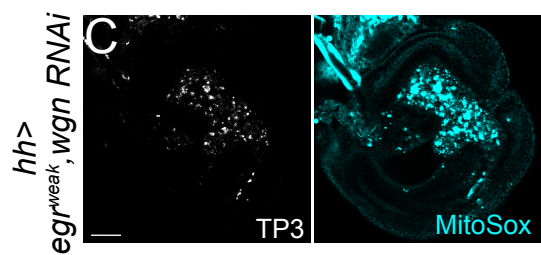
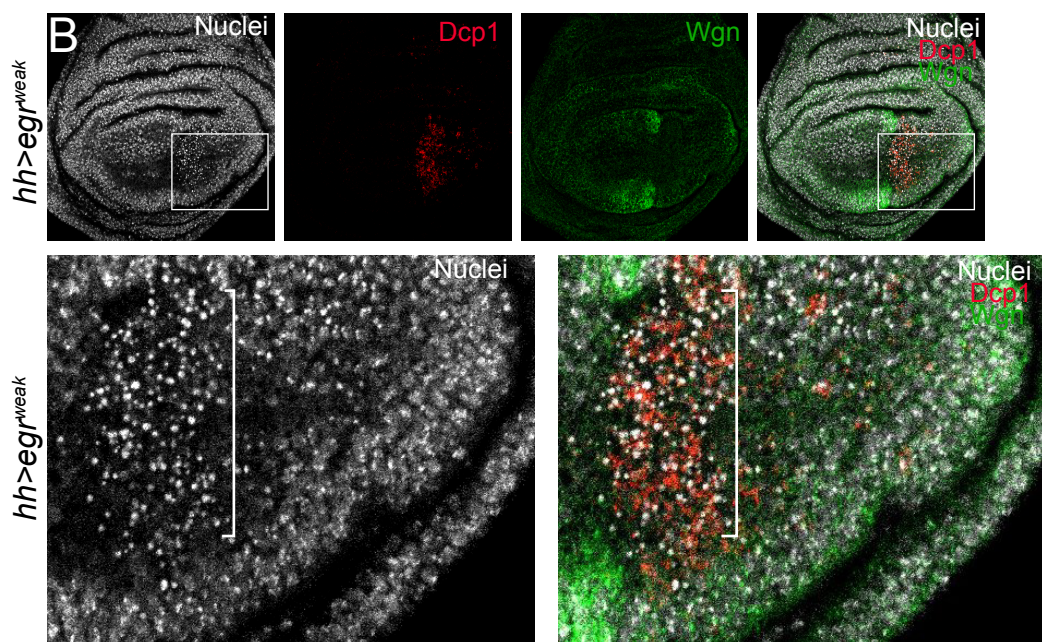
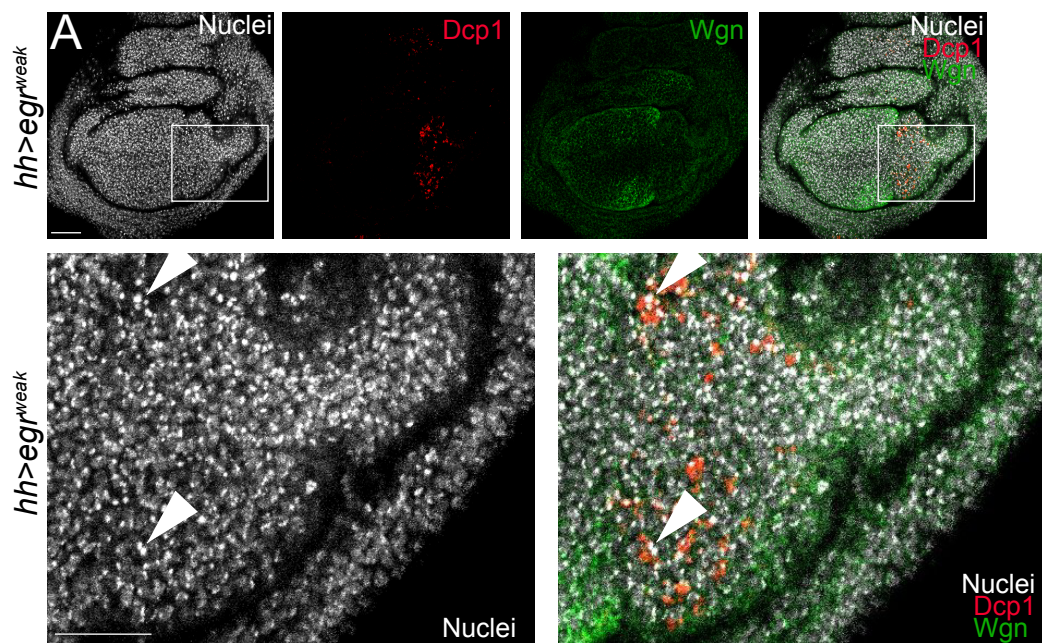


Figure Expanded View 4

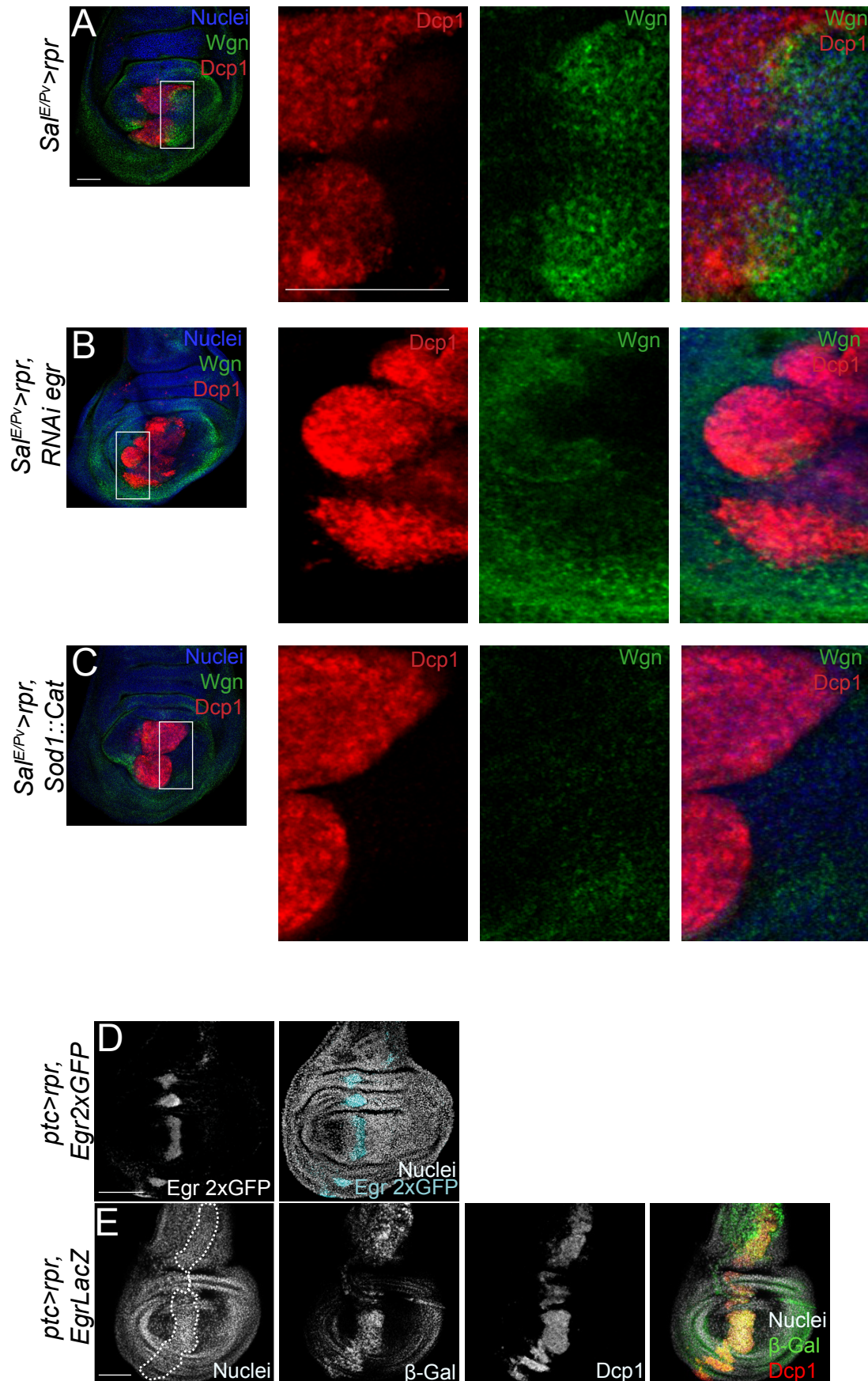


Figure Expanded view 5

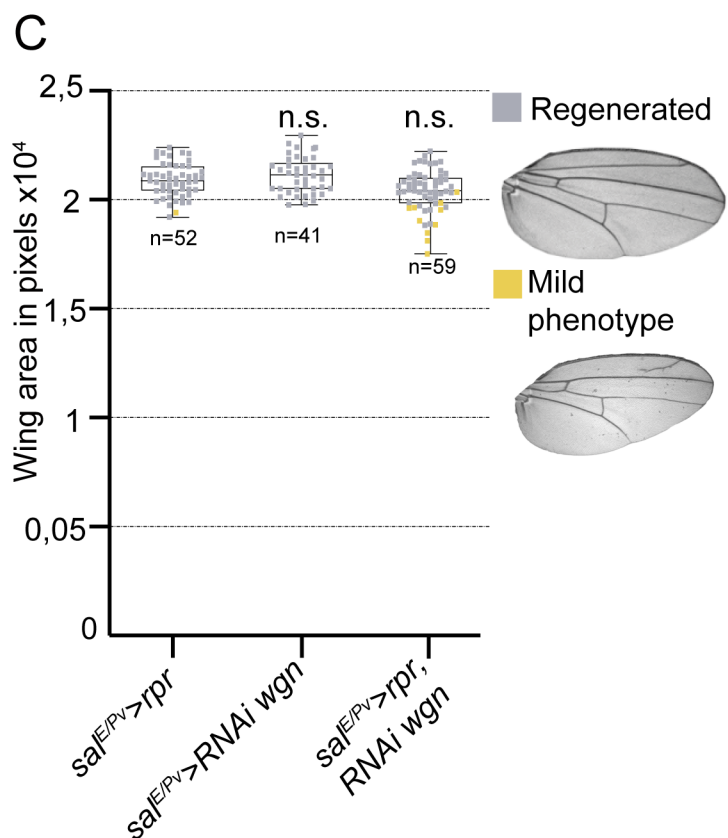
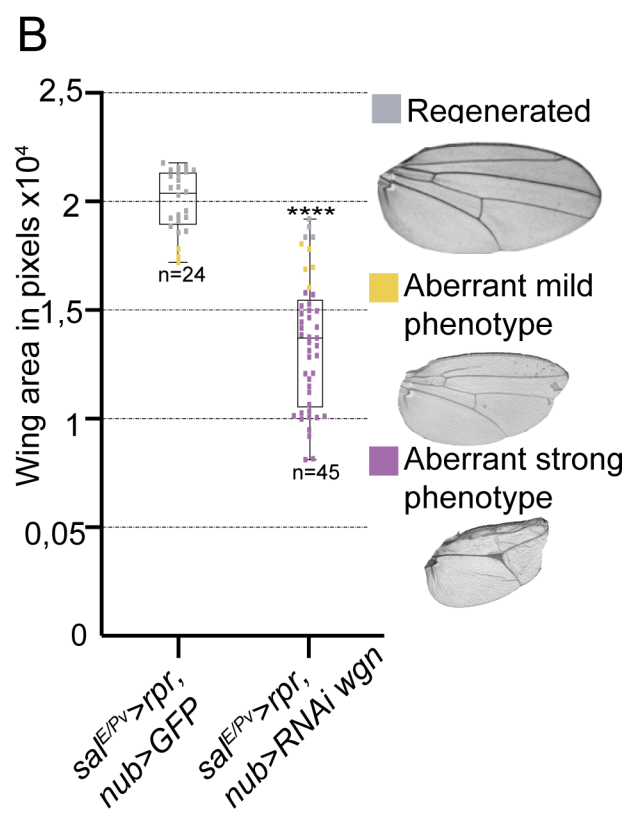
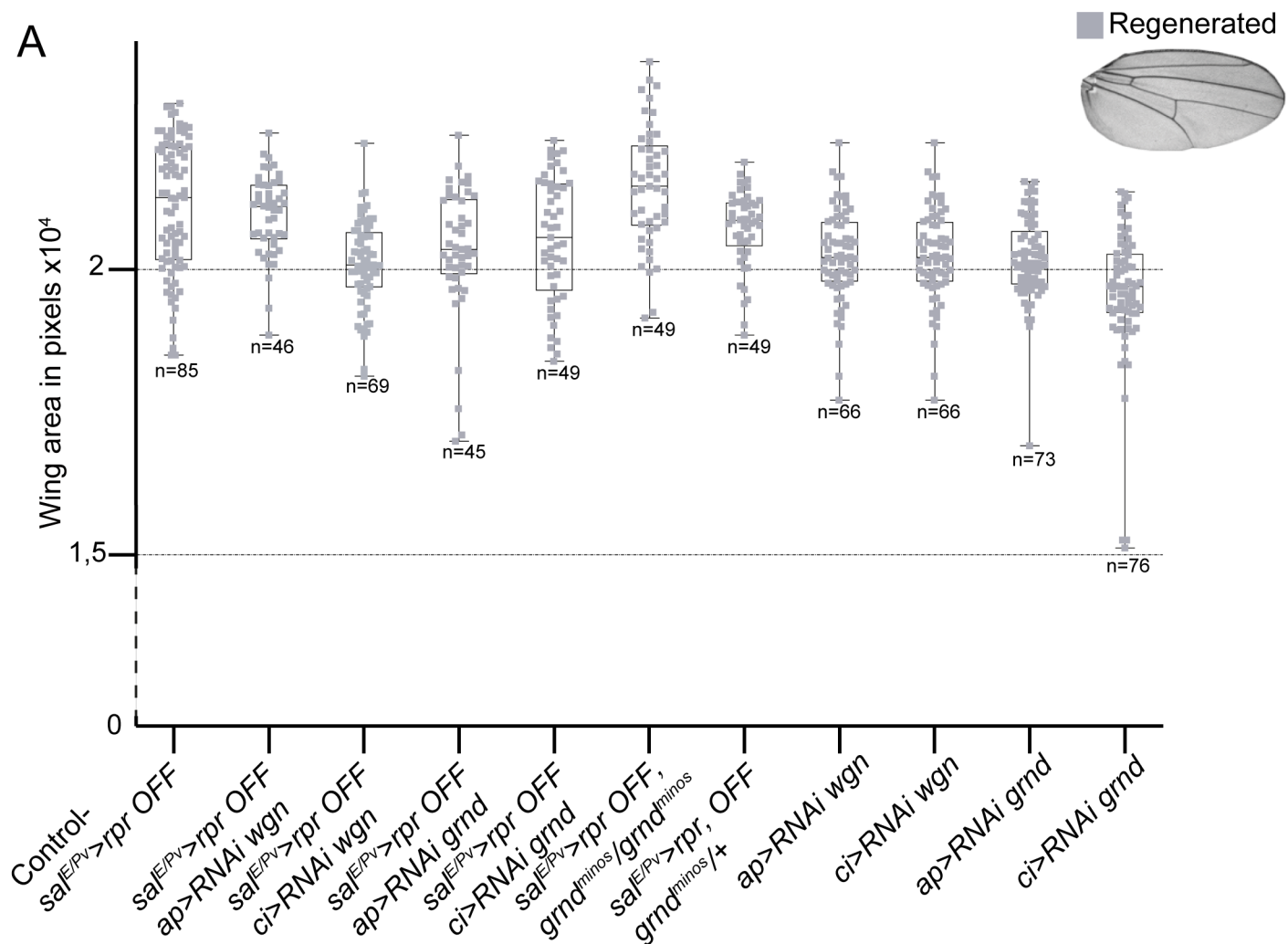


Figure Expanded view 6

General Discussion

*“La **suerte**, como suele decirse, favorece a los **tontos**”*

Hayao Miyazaki, La princesa Mononoke, 1997

1. Ask1 as a molecular hub for regeneration

1.1 Different states of Ask1 activation modulate kinase activity

A crucial question to understand regeneration is how the tissue in close proximity to the damaged area is able to sense the insult and initiate a fast response. Here, we describe an epicenter, the MAP3K Ask1, integrating different inputs from the responding cells that modulates its activity towards the initiation of a regenerative response. The nature of the signals impacting Ask1 are different in dying vs. living responding tissue. We have demonstrated that in living cells Ask1 activity is regulated by ROS-dependent Akt phosphorylation of Ser83 of Ask1. On the contrary Ask1 operates in an Akt-independent manner in the apoptotic cells.

In our study, we demonstrated that the KD domain of Ask1 is needed for p38 activation in the living cells. We speculate that phosphorylation of Ser83 induces a conformational change in the active site of Ask1, making it more accessible for Lic (MAP2K of p38) rather than Hep (MAP2K of JNK) (Lopez *et al.*, 2020). The concept of Ask1 attenuation has been previously used to describe a decrease in JNK-Ask1-dependent activation of JNK after phosphorylation of Ser83, particularly in mammals (Kim *et al.*, 2001). However, from our results, we conclude that Ask1 function is rerouted to p38 phosphorylation rather than JNK after phosphorylation of Ser83.

When a signaling pathway shifts its function toward another activity, it is often termed "pathway rewiring" (Bandyopadhyay *et al.*, 2010). Essentially, the Ask1 pathway is being diverted from its usual course to serve a different purpose. Based on our observations, we propose to consider the shift of Ask1 function from JNK to p38 as a pathway rewiring instead of attenuation.

Interestingly, in living cells surrounding the damaged area, phosphorylation of Ser83 Ask1 prevails over P-Thr. This has an impact in rewiring cells towards survival instead of apoptosis: P-Thr Ask1 results in high levels of JNK activity and apoptosis, whereas P-Ser83 Ask1 ensue p38 activation, cell survival and compensatory proliferation.

1.2 Consequences of ROS-dependent post-translational modifications of Ask1

ROS is one of the early signals released by dying cells that impacts Ask1 activity. ROS provokes the dissociation of Trx from Ask1 facilitating its oligomerization (fig. 12). Studying the different states of Ask1 activity in Trx mutant background would help to better understand the role of Trx as negative regulator of Ask1 (Fujino *et al.*, 2007).

Notably, in apoptotic cells, where high levels of general ROS are detected (Santabárbara-Ruiz *et al.*, 2015), Ask1 P-Thr levels are associated with JNK activation, as described for human Ask1 (Bunkoczi *et al.*, 2007).

In mammals, phosphorylation of Ask1 in Ser83 by Akt1 results in moderate levels of Ask1 activity (Kim *et al.*, 2001). *Drosophila* Ask1 lacks this residue (Human Ask1 Ser83) and the Akt consensus motif in the N-terminus. However, we described that the longer isoform Ask1-RC of *Drosophila* contains a conserved domain of unknown function DUF4071 in the N-terminus (described in PFAM, PF13281) with a highly conserved Ser

residue in position 83 (corresponding to human Ser174), which we hypothesized that could have a similar function as Ser83 in mammals. Indeed, the Ser83 residue in the DUF4071 domain showed a putative non-canonical Akt1 phosphorylation site (ILTQQRPLSYHYGVRESF). Both Ser (Ser75 and Ser83) residues of this sequence are spatially exposed in a 3D spatial reconstruction similarly to human Ser83 of Ask1, which would make this Ser83 residue more accessible to kinase phosphorylation events in flies (unpublished data).

We speculate that low levels of ROS (Santab rbara-Ruiz *et al.*, 2015) can be impacting Ask1 activity in two ways in living cells: (1) Less molecules of Trx detaching from Ask1 (compared to dying cells); leaving many Ask1 in a non-oligomerized form and therefore unable to auto-phosphorylate; and (2) Akt1 phosphorylation of Ser83 residue, resulting in rewiring the pathway towards p38 function and a regenerative response.

This hypothesis raises a fundamental question: Can phosphorylation events on Thr and Ser83 occur simultaneously, or are they mutually exclusive within the same group of cells? Ectopic activation of *akt* and overexpression of a *UAS-Ask1^{WT}* form leads to elevated levels of P-Ser83, while the phosphorylation of Thr is abolished (see Appendix 1). Conversely, ectopic *akt* activation alongside the overexpression of a mutated form of *Ask1*, *UAS-Ask1^{S83A}* (where Akt-mediated phosphorylation of Ser83 is hindered), results in elevated levels of P-Thr. These findings suggest that both phosphorylation events do not occur simultaneously. Moreover, it appears that P-Ser83 induced by Akt is adequate to prevent autophosphorylation of the Thr residue in *Drosophila* (see Annex1).

1.3 Akt modulation of Ask1 activity

A role for Akt on Ask1 function is to suppress Ask1-induced apoptosis. There are evidences in the literature of Ask1 phosphorylation by the PI3K/Akt pathway could reduce apoptosis induced by transfected Ask1 in 293 and HeLa cells (Kim *et al.*, 2001). In superior cervical ganglion neurons, which die in an Ask1-dependent manner upon withdrawal of nerve growth factor, nerve growth factor-induced survival may reflect activation of Akt and subsequent inhibition of Ask1 (Crowder and Freeman, 1998; Kanamoto *et al.*, 2000). So, it seems plausible that the cell death induced by Ask1 may, therefore, be prevented by simultaneous Akt activation and that this mechanism is evolutionary conserved.

The JNK and p38 signaling pathways can trigger or modify gene transcription by switching on various TFs, like members of the AP-1 family and ATF-2, with different consequences (Tibbles and Woodgett, 1999). For example, in PC12 cells, Ask1, has been observed to trigger cell differentiation by activating p38 (Takeda *et al.*, 2000). Then, we suggest that Akt's regulation of Ask1 could add another layer to its involvement in controlling gene expression during stress responses. We propose that the impact of Akt on Ask1's function could vary depending on Ask1's arrangement and its interaction with other molecules, such as the TRAF adaptor proteins, in specific situations.

Some evidences point to the N-terminal region of MAP3K Ask1 as critical in its regulation, further confirming the importance of this region, whose deletion results in a constitutively active form of the protein, as demonstrated in mammals (Saitoh *et al.*, 1998). TRAFs proteins, such as mammalian TRAF2/6 activate Ask1, in part, through Ask1 dimerization (Gotoh and Cooper, 1998; Nishitoh *et al.*, 1998; Hoeflich *et al.*, 1999; Liu *et al.*, 2000). Both Trx and TRAF2 have been shown to interact with the Ask1 N-terminal

part in mammals, a region that contains the Akt phosphorylation site. Akt post-translational modification of Ask1 may therefore modify the affinity of Ask1 for TRAF2 or Trx binding. On the other hand, it could be speculated that Akt phosphorylation cannot happen unless Trx dissociation from Ask1 occurs first.

1.4 TRAFs association with Ask1 during damage response

Previous results suggest that the TRAF2/Ask1 interaction requires previous oxidant-mediated dissociation of Trx from Ask1, which triggers Ask1 oligomerization (Liu *et al.*, 2000). We demonstrated here by knocking down TRAF1 and Ask1 that both proteins operate in the same axis upon genetic ablation. We speculate that oxidative stress would be directly implicated in TRAF1/Ask1 association by ensuring Trx dissociating from Ask1, but also by ensuring TRAF1 interaction with Ask1. This is supported by the observation that TNFR Wgn interacts through its intracellular domain with TRAF1, preventing its degradation in the proteasome (Geuking, Narasimamurthy and Basler, 2005). As shown in Chapter 3, ROS-mediated Wgn accumulation could stabilize TRAF1 and promote functional TRAF1/Ask1 complexes.

1.5 Ask1 may modulate the proliferative response in imaginal discs

Our results point to a key function of Ask1 ensuring the compensatory proliferation response during the regeneration, but not during normal development of the disc. Interestingly immunostaining against P-Thr shows co-localization with mitosis in imaginal discs, correlating with previous evidences that Ask1 has a role in cell cycle related with the G2/M transition and correct spindle orientation and positioning (Yamamoto, Ichijo and Korsmeyer, 1999; Luo *et al.*, 2016). Cell Division Cycle 25C (Cdc25c) is a dual-phosphatase implicated in the progression of the cell cycle, and has been described to be a negative interactor of Ask1 by inhibiting its oligomerization and dephosphorylating P-Thr when cells are in interphase. Moreover, Cdc25 overexpression suppresses oxidative-stress dependent activation of Ask1 (Zou *et al.*, 2001). Given these evidences, Cdc25c was expected to inhibit Ask1 activation in interphasic cells. We have preliminary data (not shown) that points to P-Thr levels change in imaginal discs after overexpression or downregulation of *cdc25c*, meaning that Ask1 would be implicated in the proliferation response in *Drosophila* through the regulation of the cell cycle. Another mechanisms would include the activation of p38 pathway which has a contrasted role in cell proliferation responses (Nebreda and Porras, 2000), but not directly controlling cell cycle through Cdc25.

1.6 Ask1 in stress induced disorders

It is pretty well established that Ask1 play pivotal roles in pathologies in which ER stress and ROS are common pathogenic factors. On one side, Ask1 prevents stress-induced disorders and protect the whole body from viral and bacterial infection under physiological circumstances (Dunnill *et al.*, 2017). On the other side, Ask1 causes adverse effects, possibly through the aberrant induction of cellular apoptosis, differentiation and the exacerbated production of inflammatory cytokines, under some pathological conditions, such as those found in inflammatory diseases, and chronic inflammation-induced carcinogenesis (Hayakawa *et al.*, 2011a, 2012). p38 inhibitors have received

much attention as therapeutic approaches to these diseases. Conversely, these drugs have high toxicity and insufficient clinical efficacy (Cohen and Fleischmann, 2010). It is tempting to speculate that this limited efficacy could be due to the inhibition of the anti-inflammatory properties described for p38 through production of anti-inflammatory cytokines (Guo, Gerl and Schrader, 2003). Alternatively, Inhibition of the p38 pathway could result in the upregulation of antagonistic pathways, such as the JNK pathway (Wagner and Nebreda, 2009), in some cellular contexts where has also been implicated in Rheumatoid Arthritis (RA) pathogenesis (Hammaker and Firestein, 2010). Targeting Ask1 may be an alternative therapeutic approach because inhibition of Ask1 efficiently suppresses stress-induced activation of both: p38 and JNK. The development of new drugs targeting specifically the N-terminal region of Ask1 would be very helpful, not only suppressing its functions, but modulating its activity towards the JNK or p38 pathway in different pathogenic contexts.

2. IIS and PI3K/Akt pathway in regeneration

2.1 Towards a molecular mechanism for ROS-dependent activation of the PI3K/Akt pathway

We have demonstrated that P-Akt, as reporter of IIS activity and nutritional status, increases notably in living cells responding to damage in a ROS dependent manner. An intriguing question arises: How does ROS target and activate the PI3K/Akt pathway in stressed cells?

One putative molecule to sensitize ROS in the PI3K/Akt pathway is the tumor suppressor Pten. Pten is frequently mutated or deleted in various human cancers (Fusco *et al.*, 2020). In mammalian cells, Pten is inhibited by oxidation of Cys residues (Cys124 and Cys71) near the catalytic site (Leslie *et al.*, 2003a). These Cys residues are very well conserved throughout evolution, and corresponds to Cys79 and Cys132 in *Drosophila* Pten (unpublished data).

In human Pten, the formation of a disulfide bond between Cys124 and Cys71 induces a conformational change that disrupts the substrate binding site, thereby abolishing the catalytic activity of the protein (Salmeen and Barford, 2005). It would be intriguing to determine whether ROS targets Pten in *Drosophila* and to define the role of Pten in ROS-dependent regeneration. Preliminary results show that regeneration is impeded in *Drosophila* wing imaginal discs upon overexpression of Pten. Interestingly, this effect can be rescued by oxidative stress (adding H₂O₂ to the fly food of ablated larvae) in a dose-dependent manner (see Appendix 2). However, the oxidation of residues Cys79 and Cys132 by ROS in *Drosophila* as the molecular mechanism inactivating Pten during the regeneration process deserves more attention, as a positive answer would reveal an evolutionarily conserved mechanism to modulate the Insulin pathway under stress conditions.

2.2 p38 response in regeneration is influenced by PI3K/Akt

The conserved IIS and the mTORC1 pathways are key in homeostasis, stress response, growth and ageing. Reduced activity of IIS and mTORC1 can impair wound healing in

flies and mice (Squarize *et al.*, 2010; Kakanj *et al.*, 2016). We have shown that mutation of the Ser83 Ask1, the target amino acid of PI3K/Akt, reduces p38 phosphorylation and impairs regeneration. Interestingly, we also found that after nutrient restriction, regeneration was compromised. Remarkably, we have shown that regeneration can be recovered by ectopic activation of p38 pathway in imaginal discs.

Diabetic mice fail to progress from the inflammatory to proliferative phase of regeneration because of the sustained activation of p38 (Madhyastha *et al.*, 2012; MacLeod and Mansbridge, 2016; Wu *et al.*, 2020). Drug inhibitors of p38 have demonstrated anti-inflammatory effects, primarily by inhibiting the expression of inflammatory cytokines and regulating cellular traffic into wounds, improving wound closure in diabetic mice (Medicherla *et al.*, 2009). It would be interesting to further study the involvement of P-Ser83 of Ask1 event under pathological conditions, such as diabetes, where would result in a p38 pathway deleterious over-activation.

2.3 A trade-off between regeneration and lifespan

These observations contrast with the finding that nutrient restriction promotes longevity and improves health in mammals (Colman *et al.*, 2009; Mattison *et al.*, 2017) flies (Piper and Partridge, 2018) and worms (Kenyon, 2011). Obviously, an optimal longevity for an organism would be the one that is able to combine longer life with retained capacity to respond to an insult or stress. And, as far as under starvation conditions regeneration seems to be impaired, we question if this ability could be rescued somehow in aged animals. Therefore, we speculate that p38 ectopic activation during regeneration could revert the low regeneration capabilities in starved animals.

Also, stimulation of regeneration by mechanisms dependent on stressors could affect longevity, mainly because activity of Akt can antagonize the expression of the longevity gene FOXO. FOXO gained notoriety for its capability to double lifespan in the absence of *daf-2* (the gene encoding the worm insulin receptor homologue) (Rodriguez-Colman, Dansen and Burgering, 2023). Therefore, it is likely that individuals exposed to oxidative stress, as the ROS detected in apoptotic cells after genetic ablation, will shorten lifespan by Akt repression of FOXO. It would be interesting to show if inducing regeneration results in shortening lifespan in *Drosophila*, and therefore whether there is a real trade-off between regeneration and aging.

2.4 PI3K/Akt pathway and sex-differences in regeneration

An interesting observation from the results section Chapter 2 is that female flies regenerate better than males. Flies with the genotype *Sal^{E/Pv}>rpr, nub>GFP* raised in normal food (Fig. 3E-F, from chapter 2) are able to regenerate a normal wing in 98% of the cases in females, while in males this percentage drops to the 89%. Under starvation conditions regeneration is poorer, though females regenerate better than males (69% females, 57% males) (Fig. 3 M-L, chapter 2). Sex differences have been observed in tissue and organ homeostasis, and in the capacity for repair. For example, sex difference in organ repair have been reported in studies on intestinal stem cells (ISC) in the *Drosophila* gut. Under normal physiological conditions, the proliferation of female ISCs is ~400-800% higher than the proliferation of male ISCs (Hudry, Khadayate and Miguel-Aliaga, 2016; Regan *et al.*, 2016; Ahmed *et al.*, 2020). This difference exists upon ingest of detergent as a stressor to damage the intestinal epithelium (Hudry, Khadayate and Miguel-Aliaga, 2016; Ahmed *et al.*, 2020). Also, upon oral bacterial infection (Regan *et*

al., 2016; Ahmed *et al.*, 2020), and maintained throughout life (Regan *et al.*, 2016). This is due to intrinsic ISC sexual identity, and is further enhanced in females following a mating-induced increase in ecdysone. Sex differences in ISCs and intestinal homeostasis are also described in mice (Zhou *et al.*, 2018). It would be interesting to test if in imaginal disc regeneration the proliferation response is different in females vs. males. But, as far as compensatory proliferation in discs is not stem cell-based, we cannot conclude that these sexual differences would be governed by the same mechanisms described for ISCs.

Notably, diverse animal's show female-male differences in IIS regulation and function. In *Drosophila*, IIS activity is higher in late third-instar female larvae than in age-matched males. Increased secretion of *Drosophila* Ilp2, among other factors, contribute to increased IIS activity in females (Rideout, Narsaiya and Grewal, 2015; Millington, Brownrigg, Basner-Collins, *et al.*, 2021). Females also have increased stimulation of Ilp2 secretion (Millington, Brownrigg, Basner-Collins, *et al.*, 2021) and low levels of negative protein interactions with Ilp2 that inhibit its binding with the insulin receptor (Arquier *et al.*, 2008; Honegger *et al.*, 2008; Millington, Brownrigg, Chao, *et al.*, 2021). Thus, there is a robust difference in IIS activity between the sexes in *Drosophila* larvae. It is described that sex determination gene *transformer* contributes to the female-male differences in Ilp2 secretion (Rideout, Narsaiya and Grewal, 2015; Millington, Brownrigg, Basner-Collins, *et al.*, 2021). The temporal window in which we induce genetic ablation to force regeneration in our experiments coincides with the L3 larval stage. So, an increased basal activity of IIS in females vs. males at this point would explain higher regeneration ratios in females in our results. We speculate that a higher basal IIS activity in females would result in higher activity of its PI3K/Akt pathway, making it more prone to respond to low levels of ROS detected in the responding cells, resulting in fast increase of P-Akt levels (compared to males) that would ensure the p38 regenerative response. Immunostaining quantification or Western-blot analysis of P-Akt levels detected in male vs. female imaginal discs after damage could contribute to decipher if this is really the mechanism guarantying better regeneration ratios in *Drosophila* females.

3. Damage response through TNFRs

3.1 Transcriptional regulation and cytoskeleton re-arrangements during ROS-mediated Wgn activation

We have described that TNFR Wgn in *Drosophila* is activated by ROS in a TNF α independent manner in response to apoptosis. The transcriptome from regenerating imaginal discs (Vizcaya-Molina *et al.*, 2018) showed an increase of *wgn* occurring after genetic ablation. Although the mechanism remains unsolved.

A redistribution of Wng positive vesicles upon damage could also explain the accumulation of Wgn in the responding cells. Intracellular trafficking is highly dependent on microtubules and actin microfilaments. It is described that ROS can target tubulin and actin dynamics to regulate the tracks for intracellular trafficking. Physiological levels of ROS are required for correct microtubule and F-actin dynamics both *in vitro* and *in vivo*. Oxidative stress induces the dissociation of F-actin and prevents microtubule polymerization. For instance, oxidation of Met 44 in F-actin results in severing of the actin filaments (Hung, Pak and Terman, 2011). Also, down-regulation of ROS synthesis, mainly through NOX inhibition, decreases the F-actin content and alters the dynamic properties of the actin cytoskeleton. In microtubules, α -Tubulin and β -tubulin contain

12 and 8 Cys residues, respectively, and both are described to suffer oxidation by endogenous and exogenous oxidizing agents (Luduena and Roach, 1991a; Löwe *et al.*, 2001; Landino *et al.*, 2002, 2004). The biological function of these Cys residues are related to GTP binding and microtubule polymerization (Mellon and Rebhun, 1976; Luduena *et al.*, 1985; Luduena and Roach, 1991). *In vitro* polymerization assays shows that oxidative species rescues tubulin polymerization (Landino *et al.*, 2007). Drug treatment during larval stages with chemicals that alter normal cytoskeleton dynamics (such as mitomycin C) would reveal if the distribution of Wgn positive vesicles is altered in a cytoskeleton-dependent manner. It would be interesting to further explore the implication of changes in cytoskeleton dynamics in response to low levels of ROS detected in living cells in the regenerative of imaginal discs, and more concretely, in the accumulation of Wgn positive vesicles in the responding cells.

3.2 CRD and its role in self-association of TNFRs to promote ligand-independent responses

A hallmark common to all TNFR is the presence of the CRD at the extracellular region, consisting of six cysteines that form three disulfide bridges. It has been shown that oxidative stress promotes TNFR receptor self-interaction in human TNFR1 and TNFR2, resulting in ligand-independent and enhancing ligand-dependent TNF α signaling (Ozsoy *et al.*, 2008). Classic models describing TNFR signaling points to TNF α recruiting three separate chains of the TNFR through ligand-induced trimerization of monomeric receptor chains, which in turn results in the recruitment of signaling complexes to the cytoplasmic domains (Banner *et al.*, 1993; Smith, Farrah and Goodwin, 1994; Hsu, Xiong and Goeddel, 1995). However, other studies suggest that members of the TNFR superfamily exist as pre-assembled trimers prior to ligand stimulation (Chan, Siegel and Lenardo, 2000). Indeed, the CRD may be critical for forming a pre-ligand assembly domain that mediates TNFR self-association in the absence of ligand binding (Siegel *et al.*, 2000). In *Drosophila*, TNFR Wgn and Grnd also interacts with TNF α through trimerization (Palmerini *et al.*, 2021). It would be intriguing to further asses if ROS exposure can induce self-assembled trimers of Wgn in the absence of the ligand. Furthermore, mutants of Wgn in which key Cys residues of the CRD were changed by a hydrophobic residue (unable to undergo oxidation) would help to further study the involvement of Cys in the final 3D structure of TNFR Wgn. And, also, if they have any functional relevance, in terms of TNFR signaling activation, in response to ROS appearance.

Remarkable, TNFR1 and 2 from human and Wgn from *Drosophila* can trigger ligand-independent TNF signaling in response to ROS. Remarkably, TNFR1 and 2 in humans, and Wgn in *Drosophila*, exhibit the ability to initiate ligand-independent TNF signaling in response to ROS. Given our findings indicating a high degree of similarity between Wgn's CRD and various TNFRs' CRDs from deuterostome species, including human TNFR1, we hypothesize that this mechanism could represent an evolutionarily conserved pathway to activate TNF α signaling in response to damage or apoptosis.

3.3 Regeneration responses triggered by TNFRs are evolutionary conserved

In other species, for example in hydra, a TNFR protein (TNFR16) is found expressed at the tips of the wound after amputation, suggesting it could be involved in signal

transduction during wound healing (Wenger *et al.*, 2014). Also, it has been shown that injury-induced TNF mediated responses take place during liver regeneration in mice after partial hepatectomy (Diehl *et al.*, 1994), and knocking out TNFR1 in mice impairs DNA synthesis and liver regeneration (Yamada *et al.*, 1997). In this thesis we show that Wgn is in charge of the p38-dependent regenerative response in the wing imaginal disc of *Drosophila*. All these evidences point to the involvement of TNFRs in the regeneration process, that could be also an evolutionary conserved trait of these protein family.

The CRD has deep evolutionary origins and have been described in the proteomes of sponges (Srivastava *et al.*, 2010), Choanoflagellates (King *et al.*, 2008), Cnidarians (Shinzato *et al.*, 2011), Ctenophores (Moroz *et al.*, 2014), and plants (Nikonorova *et al.*, 2015). To date, the Choanoflagellates species have the highest number of CRD-containing proteins (Quistad and Traylor-Knowles, 2016). Interestingly, none of these CRD-containing proteins possess DD, suggesting that these TNFRs are not involved in apoptosis. It has been proposed that CRD appeared in the last common eukaryotic ancestor followed by a fusion event with a DD during the last common metazoan ancestor that would result in the first TNFR involved in apoptosis.

Wgn TNFR lacks a DD domain and, although it has been linked in the literature with cell death response in some contexts (Moreno, Yan and Basler, 2002), there are many evidences of its involvement in many non-apoptotic functions. For instance, we demonstrated that ROS-dependent activation of Wgn is in charge of the p38-dependent regenerative response, a pathway widely implicated in cell survival and proliferation response in tissues. We can find in the literature other examples of TNFRs exerting important roles in response to stress, different from cell death. For instance, regulating immune responses under abiotic stress exposure (environmentally-equivalent conditions of temperature, salinity and desiccation), in the case of the pacific oyster (*Crassostrea gigas*) (Zhang *et al.*, 2015). Furthermore, our phylogenetic analyses using the CRDs shows that Wgn branch include genes from multiple arthropod groups, from dipterans to chelicerates, indicating that Wgn has a very ancient origin around ~546mya. All these non-apoptotic functions described for Wgn would indicate an ancient trait of this TNFR in *Drosophila*.

Our results point to Grnd having a more restricted taxonomic distribution, only present in insects and crustaceans, suggesting a slightly younger origin in evolution than Wgn. In addition, Grnd is an evolutionary novelty, taxonomically restricted to Arthropoda (regarding the low degree of similarity with TNFR from deuterostome species). We can conclude that Grnd and Wgn are very different from an evolutionary perspective, suggesting an independent origin of both TNFRs.

3.4. Functional relevance of Wgn and Grnd differential interaction with TRAFs

Grnd function has been linked with canonical TNF α signaling in *Drosophila*, its activation results in high levels of JNK signaling and cell death (Andersen *et al.*, 2015a). Our results further confirm that involvement of Grnd in the cell death response. Intriguingly, Grnd lacks an intracellular DD. It deserves further analysis if the interaction with different downstream partners could explain these different functions. Notably, in our study we demonstrate that Grnd seems to act through association with the adaptor molecule TRAF2 and TAK-1 MAP3K, whereas Wgn seems to function through TRAF1 and the activation of Ask1. Up to date, three different TRAFs have been described in *Drosophila*,

TRAF1, TRAF2 and, more recently, TRAF3. There are experimental evidences about the association of TRAF2 to both: Grnd and Wgn (Geuking, Narasimamurthy and Basler, 2005; Andersen *et al.*, 2015), whereas for TRAF1 these evidences only exist for TNFR Wgn (Geuking, Narasimamurthy and Basler, 2005). Moreover, a recent study in *Drosophila* gut shows that Wgn inhibits autophagy-dependent lipolysis by restricting association with TRAF3, while, in the same tissue it suppresses immune processes through inhibition of the TAK-1/TAK1-Relish/NF- κ B pathway in a TRAF2-dependent manner (Loudhaief *et al.*, 2023). Taking all these into account, we speculate that TNFR Wgn has a much wider repertoire of biological functions than Grnd, because its capability to interact with three different TRAFs that could modulate physiologically separate signaling pathways downstream.

4. Towards a model for the onset of regeneration

In this study, we present a model elucidating the initial stages of cell signaling in regeneration. Cells experiencing damage, whether through injury or genetic ablation, exhibit the production of ROS, typically stemming from mitochondrial disruption (Murphy, 2009). These ROS propagate from damaged cells to neighboring healthy cells, where they are detected at low concentrations. Notably, low levels of ROS function as signaling molecules, directly influencing proteins involved in cell signaling and transduction, typically localized at the plasma membrane or within the cytoplasm. Among the mechanisms facilitating ROS-mediated signaling, the presence of Cys residues susceptible to redox modifications plays a pivotal role in altering the three-dimensional conformation of proteins, albeit not the sole mechanism. As signaling molecules, ROS initiate tissue regeneration across various organisms, including flatworms, *Drosophila*, and mammals (Gauron *et al.*, 2013; Rampon *et al.*, 2018).

In our proposed model, ROS-dependent post-translational modifications predominantly target three signaling branches (see Fig. 12): (a) TNFR Wgn, which accumulates in responsive cells in a ROS-dependent manner, recruiting TRAF1 to the Wgn core signaling complex; (b) the insulin/PI3K/Akt signaling pathway, evidently activated by ROS (presumably through Pten redox oxidation); and (c) the Trx bound to the inactive Ask1 signaling complex, which, upon oxidation, dissociates to facilitate the appearance of the active Ask1 form.

All three post-translational modifications converge at a critical molecule: the MAP3K Ask1, serving as a central hub integrating these disparate inputs to modulate its kinase activity. (a) Trx dissociation from Ask1 induces a conformational change, leading to the activation of the protein. However, this event alone results in complete protein activation, leading to elevated levels of JNK and subsequent cell death. Thus, a fundamental question arises regarding how Ask1 activity is regulated in responsive cells following thioredoxin dissociation. (b) Recruited by Wgn, and thereby rescued from proteasomal degradation, TRAF1 is available to interact with the TRAF binding domain located at the N- and C-terminal regions of Ask1. These interactions serve two purposes: stabilizing Ask1 homo-oligomerization and acting as negative regulators to prevent complete Ask1 activation. (c) Akt, a key kinase downstream of the insulin pathway, interacts with Ask1 at its non-canonical Akt recognition site (proximal to the N-terminal

region) and phosphorylates the Ser83 residue of Ask1. This phosphorylation event prevents autophosphorylation of Thr845 in the kinase domain of Ask1, thus inhibiting JNK activation. Additionally, we speculate that this phosphorylation may influence the conformation of the active pocket of Ask1, rendering it more accessible for Lic (p38 MAP2K). Consequently, surviving cells activate a p38-dependent regenerative program downstream of Ask1, culminating in the complete restoration of imaginal tissue homeostasis.

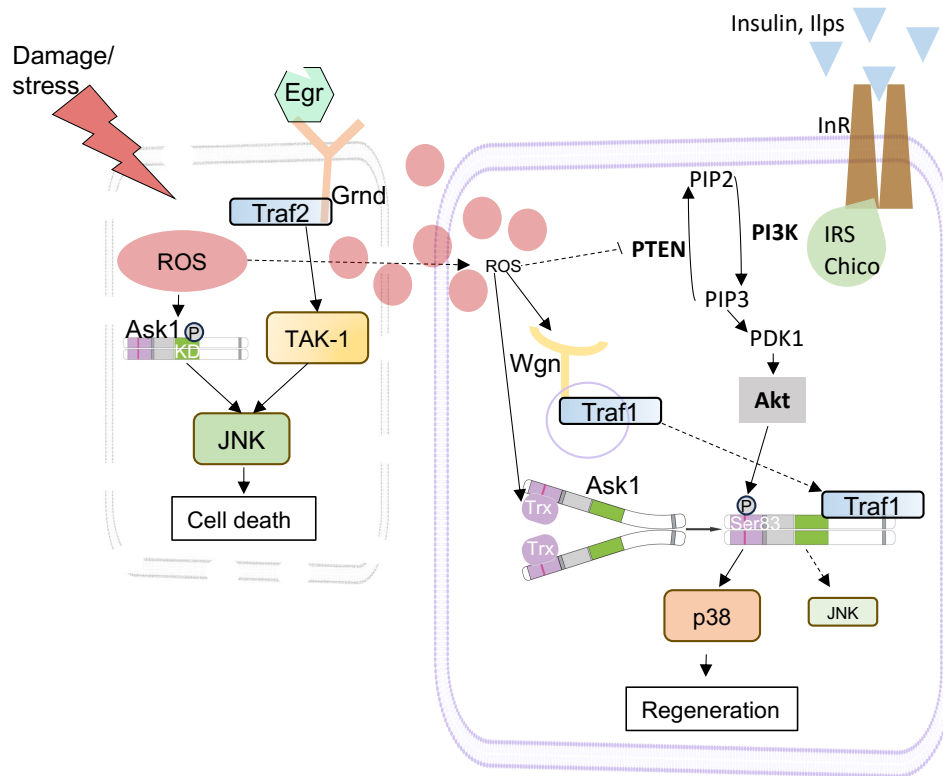


Figure 12. Model for the onset of regeneration. **Right panel:** Cell committed to die. High levels of ROS result in autophosphorylation of Ask1 in its KD. Egr released by damaged cells ensures JNK high activation via Grnd and TRAF2, resulting in apoptosis. **Left panel:** Cell committed to survive: ROS here are required for the oxidation of Trx to dissociate from Ask1. Also, for the phosphorylation of Akt, which in turn phosphorylates Ask1 Ser83 diverting its function towards p38. ROS are required for Wgn recruitment in the Ask1/p38 axis through TRAF1. Phosphorylation of Ask1 by Akt results in tolerable levels of p38 and JNK (perhaps very low, gray) activity and the avoidance of apoptosis.

Conclusions

Conclusions

Conclusions

As a summary of this thesis, we described how oxidative stress released after damage impact the SAPKs, TNF α /TNFR and PI3K/Akt pathways which share a central hub, MAP3K Ask1, that integrates inputs from all three axis, fostering p38-dependent regeneration.

1. Specific conclusions:

- Ask1 acts as an intracellular sensor that upon damage activates the JNK and p38 regenerative signaling pathways. This is controlled by PI3K/Akt which attenuates the activity of Ask1 in the living cells by residue Ser83 phosphorylation.
- Ask1 and PI3K/Akt act synergistically to respond to the stress generated after tissue damage and drive regeneration, a first overview within the framework of how insulin signaling inputs modulate the capacity to overcome tissue damage.
- Phosphorylation of Ser83 residue of Ask1 is key for activating p38, but not JNK.
- The nutritional status through PI3K/Akt signaling is crucial for p38 activation in cells involved in regeneration.
- TNFR Wgn/TRAF1 is required for cell survival, in contrast to the apoptotic role of Grnd/TRAF2.
- *grnd* and *wgn* have independent evolutionary origins, reinforcing the idea of a sub-functionalization of these genes with a higher degree of conservation between the CRDs of Wgn and the TNFRs of other deuterostomes.
- Wgn, but not Grnd, is required for p38-dependent regeneration. The activation of Wgn is Egr/TNF α -independent and is sensitive to the ROS produced by dying cells, a mechanism that could be evolutionary conserved for damage response.

2. Global conclusion:

- Regeneration is a dynamic process occurring in a physiological context which could determine the return (or not) to homeostatic conditions. Oxidative stress and the nutritional status cooperate to fine-tune SAPKs resulting in p38-regenerative response.

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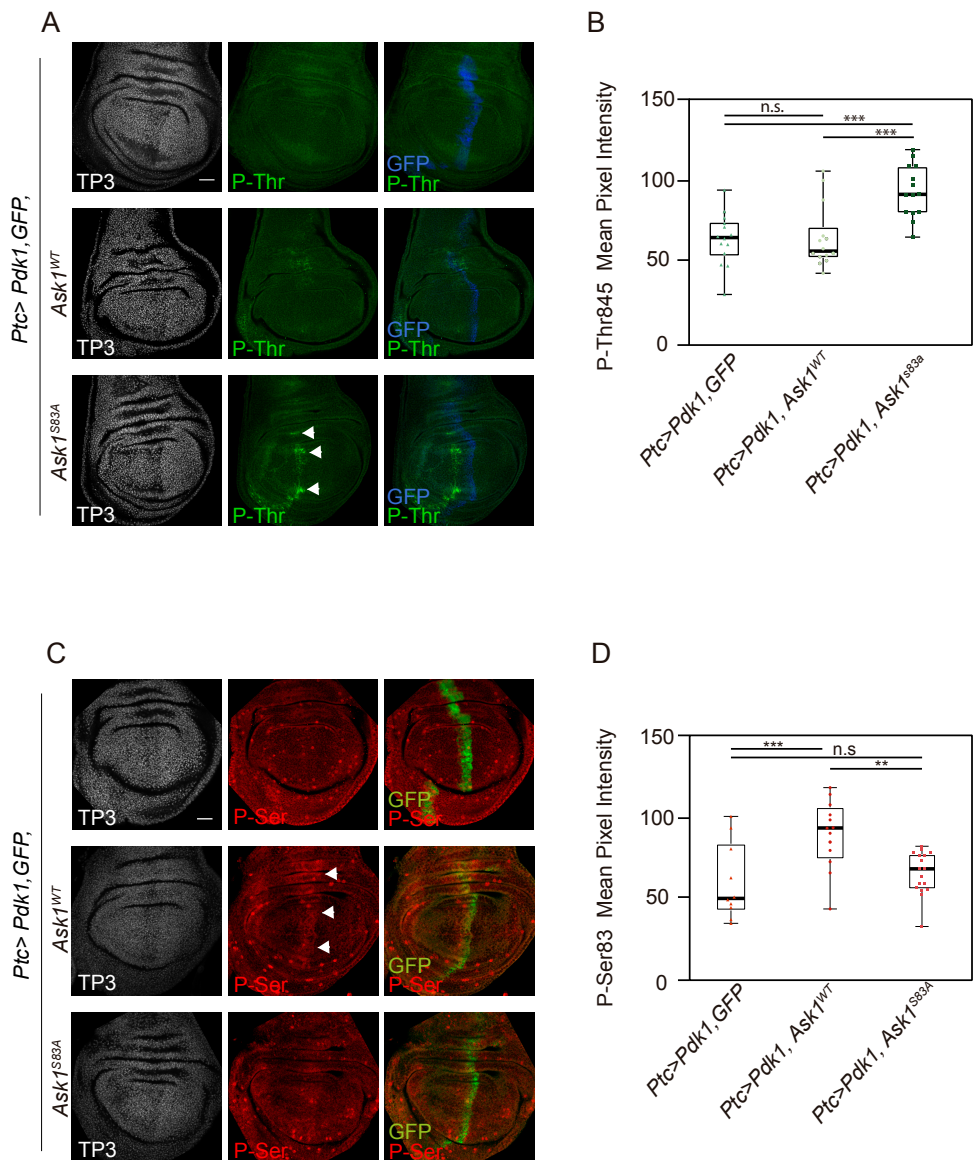
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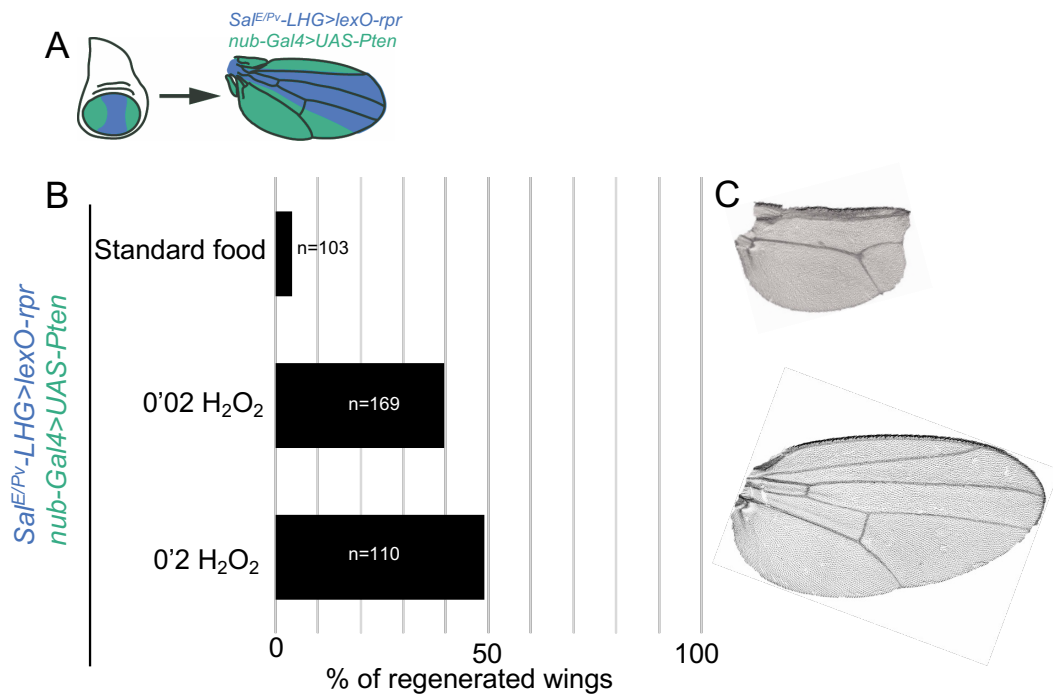
Appendix

Appendix 1



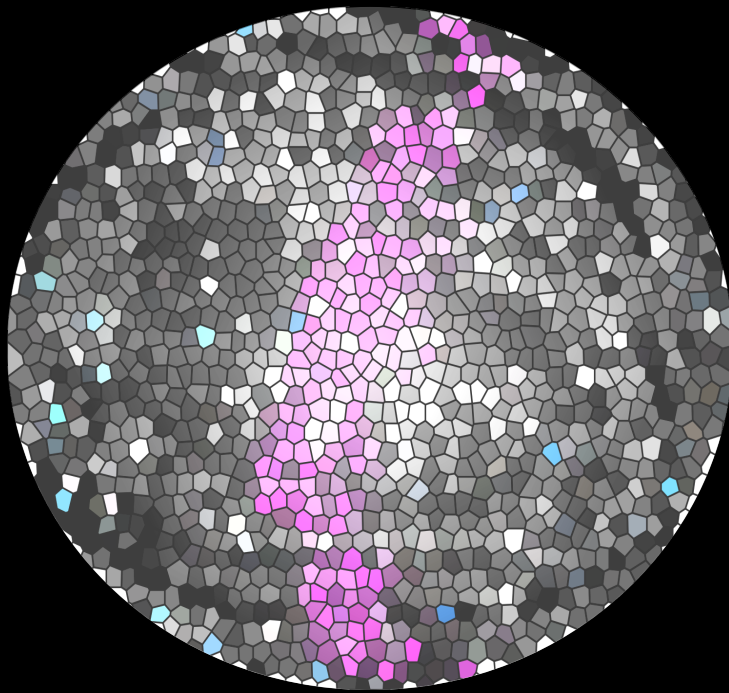
Appendix 1. P-Thr and P-Ser83 events in MAP3K Ask1 are mutually exclusive. (A) Ask1 P-Thr activity in discs co-expressing *Pdk1:Akt* and GFP-expressing control, wild-type *Ask1^{WT}*, *Ask1^{S83A}*, White arrows in *Ask1^{S83A}* outline P-Thr activation in *ptc*. (B) Mean pixel intensity of P-Thr for the genotypes indicated. (C) Ask1 P-Ser83 activity in discs co-expressing *Pdk1:Akt* and GFP-expressing control, wild-type *Ask1^{WT}*, *Ask1^{S83A}*, White arrows in *Ask1^{WT}* outline P-Ser83 activation in *ptc*. (D) Mean pixel intensity of P-Ser83 for the genotypes indicated. Box plots show maximum-minimum range (whiskers), upper and lower quartiles (open rectangles), and median value (horizontal black line). TP3 is used to stain nuclei. Scale bars: 50 μm.

Appendix 2



Appendix 2. H_2O_2 dose-dependent *Pten* inactivation rescues adult wing regeneration. (A) Cartoon of the expression domains in the double transactivation system LHG/LexO (green), Gal4/UAS (blue). (B) Percentage of regenerated wings in animals where the pro-apoptotic gene *rpr* was activated in the *Sal^{E/Pv}* domain (blue) and, at the same time *Pten* was overexpressed in the *nub* region (green) under three different conditions: normal food and food supplemented with H_2O_2 at sub-lethal concentrations. Examples of an aberrant, non-regenerated wing and a normal, fully-regenerated wing.

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