Mutual repression between JNK/AP-1 and JAK/STAT stratifies cell behaviors during tissue regeneration

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Abstract

Epithelial repair relies on the activation of stress signaling pathways to coordinate cellular repair behaviors. Their deregulation is implicated in chronic wound and cancer pathologies. Despite such translational importance, an understanding of how spatial patterns of signaling pathways and repair behaviors arise in damaged tissues remains elusive. Using TNF-α/Eiger-mediated inflammatory damage to *Drosophila* imaginal discs, we uncover that JNK/AP-1 signaling cells act as paracrine organizers and initiate a mutual repression network that spatially segregates JNK/AP-1 and JAK/STAT signaling cells into distinct populations. While JNK/AP-1 signaling cells produce JAK/STAT-activating Upd ligands, these signal-sending cells suppress activation of JAK/STAT via Ptp61F. Conversely, responding cells with activated JAK/STAT suppress JNK activation via Zfh2. The resulting bistable segregation of signaling domains is associated with distinct cellular tasks and regenerative potential. While JNK/AP-1 signaling cells at the wound center act as paracrine organizers, their cell cycle is senescently arrested. Thus, compensatory proliferation occurs exclusively in JAK/STAT signaling cells at the wound periphery. This spatial stratification is essential for proper tissue repair, as co-activation of JNK/AP-1 and JAK/STAT in the same cells creates conflicting inputs on cell cycle progression, leading to excess apoptosis of senescently arrested organizer cells. Finally, we demonstrate that bistable spatial segregation of JNK/AP-1 and JAK/STAT drives senescent and proliferative behaviors in transient as well as chronic tissue damage models, and importantly, in *Ras*^{V12}*, scrib* tumors under the influence of JNK/AP-1 activity. Revealing this previously uncharacterized regulatory network between JNK/AP-1, JAK/STAT and associated cell behaviors have important implications for our conceptual understanding of tissue repair, chronic wound pathologies and tumor microenvironments, where both pathways are strongly implicated.

Introduction

Wound repair programs rely on the coordination of distinct responses to damage [1, 2]. Upon damage, wound-derived factors initiate inflammation and stress-signaling pathways which drive cellular responses like apoptosis, proliferation, survival and tissue remodeling [3-6]. While these responses are essential, it is critical that they remain spatio-temporally restricted to avoid the establishment of chronic wounds [7-9]. Chronic wounds are characterized by sustained inflammation, and deregulated proliferation and apoptosis [10]. The finding that these are also hallmarks of tumor microenvironments supported the idea that tumors resemble chronic, non-healing wounds [11-13]. Yet, the tissue-level coordination of signaling pathways and repair behaviors upon wounding, and the conditions under which they turn pathological remain elusive.

A myriad of wound models identified specific signaling pathways required for regeneration, of which several have also been shown to drive tumor growth. In particular, the JNK/AP-1 and the JAK/STAT pathways are consistently implicated in regeneration [14-17] as well as in tumors [18-23]. In the context of wound-healing, JNK/AP-1 activation is indispensable for regeneration [24-26]. JNK/AP-1 is one of the earliest signaling responses to damage, proposedly activated by wound-derived ROS signals [27, 28]. In this role, JNK/AP-1 regulates a variety of conflicting cell behaviors including apoptosis [29, 30], survival [31] and compensatory proliferation [32, 33]. These paradoxical behaviors have been extensively characterized individually [34-36], yet how they are organized on a tissue scale to ensure regeneration is not known.

We recently reported that high JNK/AP-1 signaling facilitates survival in wounds and tumors by mediating a cell cycle stall in G2 which is characterized by anti-apoptotic and senescent features [31]. Forced exit of these cells from G2 into mitosis and G1 caused a substantial increase in apoptosis, suggestive of the JNK-mediated G2 stall being a protective state during regeneration (Fig. S1A) [31]. This model is supported by recent evidence that the universal integrator of molecular damage, p53, is activated by the G2/M kinase Cdk1 to induce apoptosis competence upon exit from G2 [37]. The G2 protective state is critical as high JNK/AP-1 signaling cells are shown to produce mitogens necessary to induce compensatory proliferation and regeneration [28, 38-40]. Paradoxically, this reveals a cell population at the center of wounds which produces pro-proliferative signals but itself is prohibited from proliferation. These findings highlight the necessity of a mechanism, which ensures that high JNK/AP-1 signaling cells maintain a G2 stall in the presence of mitogenic signals, thereby outsourcing proliferation to other cells. Alternatively, it requires the existence of a mechanism that promotes cycling as well as survival of JNK-signaling cells. This illustrates that the underlying

84 mechanisms and molecular effectors that resolve these conflicting cell behaviors downstream
85 of JNK/AP-1 signaling in the same tissues remains elusive.
86
87 Prominent cytokines produced by JNK/AP-1 signaling cells belong to the Unpaired (Upd) family
88 [41-45] and activate the JAK/STAT pathway which, in turn, plays a significant role in mediating
89 proliferation [18, 20, 28, 39, 46-50] and cell survival during tissue stress [51, 52]. Interestingly,
90 we have previously also shown that JAK/STAT is required in the disc to restrict the expansion
91 of JNK/AP-1 signaling [51]. This is essential as JNK/AP-1 signaling within the tissue can self-
92 propagate by promoting an increase in the expression of its own paracrine activators - the
93 TNF-α/Eiger (Egr) cytokine [53, 54], as well as ROS [28, 55, 56].
94
95 These observations indicate an interdependence between the JNK/AP-1 and JAK/STAT
96 signaling pathways, whose coexistence has been consistently reported in wounds [14-17] and
97 tumor tissues [18-23]. Yet the regulatory network of the two pathways, beyond the regulation
98 of *upd's* by JNK, remains so far unexplored. As JNK/AP-1 and JAK/STAT drive contradicting
99 yet critical cell behaviors - namely G2 stalling and proliferation, linked by their regulation of
100 survival - characterizing their regulatory interactions is essential to understand how these
101 pathways cooperatively organize tissue stress responses.

102 **Results**
103

104 **Figure 1**
105 **JNK/AP-1 and JAK/STAT segregate into distinct spatial domains upon tissue damage**
106
107 To characterize the JNK/AP-1 and JAK/STAT signaling pathways at wound sites in detail, we
108 analyzed signaling dynamics and repair behaviors induced by targeted expression of the TNF-
109 α homologue *eiger* (*egr*) in the wing disc pouch (Fig. S1.1B,C). Ectopic *egr*-expression has
110 been extensively used as a model to genetically induce wounds and study regeneration [57].
111 *egr*-expression activates the stress-signaling pathway JNK/AP-1, assessed by the sensitive
112 JNK/AP-1 reporter - *TRE>RFP* (Fig. 1A,B) [58], which leads to loss of polarity, epithelial barrier
113 dysfunction (Fig. S1.1D-E') and inflammation [24, 25, 59]. Specifically, expression of *egr* also
114 induces *upd1-3* cytokine expression [51], MMP-1 production [60-62], ROS production [8, 63]
115 and protective responses including UPR [64] and, ultimately, NF-κB signaling upregulation [65]
116 (Fig.S1.1F-O, see also Fig.1E,F) in high JNK/AP-1 signaling cells – which are all known
117 hallmarks of inflammatory, and by extension, chronic wounds [6, 10]. In addition, high JNK/AP-
118 1 activity at the center of the *egr*-expressing domain (Fig. 1A,B) induces a senescent-like G2
119 cell cycle stall (Fig. 1C,D) which is essential for cell survival (Fig. S1.1A), as assessed using
120 the Fly-FUCCI toolkit [31, 66]. Indeed, we found that proliferation was excluded from the

121 inflammatory JNK-signaling domain at the center of the disc and was restricted to the wound
122 periphery (Fig. 1C,D,N). These observations highlight a pronounced spatial segregation of
123 regenerative cell behaviors linked to JNK-induced tissue damage responses.
124
125 Upon tissue damage, *upd's* - the pro-mitogenic cytokines for the JAK/STAT pathway - are
126 generally upregulated at wound sites where JNK/AP-1 activity is high (Fig. 1E,F,M, see also
127 S1.1F,G) [20, 28, 49, 51]. Consistent with their paracrine function [67, 68], JAK/STAT signaling
128 was broadly induced in the pouch periphery and hinge of *egr*-expressing discs (Fig. 1G,H),
129 coinciding with regions of proliferation (Fig. 1K,L,N). Yet, high JNK-signaling cells in *egr*-
130 expressing discs that upregulated the transcriptional reporters for *upd1* and *upd3* (Fig.
131 S1.1F,G; Fig. 1E,F,M) [69] did not undergo proliferation and, importantly, did not exhibit
132 JAK/STAT activity, as assessed by the *10xStat92E>dGFP reporter* (Fig. 1I,J) [70]. This was in
133 stark contrast to wing disc development where ligand expression and pathway activation
134 patterns largely coincide [70-73]. Our unexpected finding that JAK/STAT signaling is low in
135 *upd*-producing cells, strongly suggests that a distinct regulatory network controls JAK/STAT
136 activation during JNK-driven responses to tissue damage.
137
138 To further support this conclusion, we first wanted to rule out that the observed JAK/STAT
139 activation pattern in the periphery of *egr*-expressing discs perpetuated from early hinge
140 patterns of developing discs due to a Dilp8-induced developmental delay (Fig. S1.2 A) [74].
141 Hence, we monitored temporal changes in JAK/STAT activity via a time course of 0h, 7h and
142 14h of *egr*-expression. We consistently observed ectopic induction of the JAK/STAT reporter
143 in the hinge, above developmental levels and with an altered pattern (Fig. S1.2 B-M). This data
144 confirmed that *upd1-3*-driven JAK/STAT activity is induced *de novo* in the hinge and pouch
145 periphery upon tissue damage.
146
147 In summary, we find that JNK/AP-1 activation in the *egr*-expressing pouch coincides with the
148 expression of *upd* cytokines. We show that *upd* cytokines activate JAK/STAT only non-
149 autonomously in a cell population spatially segregated from high JNK-signaling and *upd*-
150 producing cells. In addition to the well-described non-autonomous activation of JAK/STAT by
151 JNK/AP-1 activation, this strongly indicates that cell-autonomous repression of JAK/STAT
152 signaling may be an important consequence of JNK/AP-1 activation (Fig. 1O). This is
153 consistent with recent observations of JNK/AP-1 and JAK/STAT activity segregation during
154 tissue stress in other tissue types, such as epidermal wounds [14]. We therefore demonstrate
155 a recurring theme of spatial segregation of JNK/AP-1 and JAK/STAT signaling upon tissue
156 damage. Importantly, we also show that such spatial patterns of segregating JNK/AP-1 and

157 JAK/STAT signals strongly correlate with spatial segregation of regenerative cell behaviors,
158 specifically G2 stalling and proliferation, into spatially distinct signaling domains.
159

160 **Figure 2**
161 **JNK/AP-1 signaling represses JAK/STAT activity**
162

163 We wanted to first understand whether JNK/AP-1 directly represses JAK/STAT activity and
164 thereby restricts it to the wound periphery. We therefore tested if clonal expression of JNK/AP-
165 1 directly represses JAK/STAT activity, by co-expressing a constitutively activated form of the
166 JNKK Hep along with p35 to prevent cell death [75, 76]. Indeed, *hep*^{act} clones cell-
167 autonomously repressed JAK/STAT activity (Fig. 2A-D, S2A), both when non-autonomously
168 activating it in surrounding cells in the peripheral pouch, hinge or peripodium (Fig. 2A-C, S2A),
169 as well as when within developmentally patterned activity in the hinge (Fig. 2D) [72]. These
170 experiments demonstrate that JNK/AP-1 represses JAK/STAT signaling cell-autonomously,
171 even when JNK/AP-1 has the ability to activate JAK/STAT non-autonomously.
172

173 In these mosaic experiments, hinge cells activated JAK/STAT signaling more robustly than
174 cells of the central pouch (Fig. 2A-B), likely as a result of developmental pre-patterns conferring
175 distinct competence [72]. Namely, the transcription factors Nubbin (Nub) and Rotund (Rn)
176 reduce developmental Stat92E activity in the pouch [77]. To first exclude the possibility that
177 JNK/AP-1 represses JAK/STAT by inducing pouch-specific transcription factors [77], we
178 assessed Nub and Rn levels within *hep*^{act} clones. *hep*^{act} clones did not ectopically induce Nub
179 or Rn, in fact, Nub was distinctly downregulated (Fig. S2B-D). Furthermore, we expressed *egr*
180 in the posterior compartment of the wing disc and examined whether JAK/STAT could be
181 activated in the adjacent anterior pouch (Fig. S1.1B). The cells highly positive for the JNK/AP-1
182 target gene - MMP-1 [78] did not activate JAK/STAT signaling. However, JAK/STAT signaling
183 could be distinctly activated in the surrounding tissue, including the anterior pouch (Fig. 2E,F).
184 These experiments confirm that JAK/STAT signaling can *in principle* be activated *de novo* in
185 the pouch by cell non-autonomous activity of JNK/AP-1 signaling cells. Altogether, these
186 experiments demonstrate that tissue damage associated with high levels of JNK/AP-1
187 signaling repress JAK/STAT activity in a cell autonomous manner, independent of
188 developmental competence.
189

190 Accordingly, we wanted to better understand if JAK/STAT repression by JNK/AP-1 may
191 depend on JNK/AP-1 signaling strength or time. We therefore analyzed the temporal evolution
192 of JNK/AP-1 and JAK/STAT activity in the JNK-signaling domain of *egr*-expressing discs. A
193 time course analysis revealed that JAK/STAT was mildly elevated during short-term JNK-

signaling in the pouch by 7 h of *egr*-expression, and increased even further with 14 h of *egr*-expression (Fig. 2K,L,N,O, Fig. S2E,F,H,I,K,). Interestingly, cells displayed co-existence of both pathways at moderate levels, but also often displayed exclusive activation of either reporter (Fig. S2L,M). After 24 h of sustained *egr*-expression, high JNK/AP-1 signaling was observed in the pouch, but notably, JAK/STAT activity had now largely disappeared from this domain (Fig. 2M,P, Fig. S2G,J,K). These observations reveal that short-term or moderate activity of JNK/AP-1 facilitates local, intermixed activation of JAK/STAT. However, sustained and high activation of JNK/AP-1 efficiently excludes JAK/STAT activity cell autonomously. As a consequence, JNK/AP-1 and JAK/STAT signaling are increasingly segregated into distinct cell populations within the tissue.

Notably, these signaling dynamics are synchronously followed by cell behavior dynamics. We observed that prolonged JNK/AP-1 signaling correlated with an increased exclusion of proliferation from the pouch (Fig. 2G-J; Fig. 1K,L,N). Altogether, these data suggest that chronic and high levels of JNK/AP-1 activation drive spatial wound site signaling and cell behavior patterns from a local intermixed state to a larger, segregated and bistable field.

Figure 3
JNK/AP-1 acts as a wound organizer by employing a mutual-repression network

We wanted to understand the type of regulatory interactions between JNK/AP-1 and JAK/STAT pathways that establish spatially segregated signaling domains on a tissue scale [79-82]. Regulatory networks describe how components of different signaling pathways interact [83-85] or are integrated to define a cell's signaling state and establish distinct cellular outcomes [86, 87]. How regulatory networks can also organize cellular behaviors to influence tissue-level outcomes has been extensively described using mathematical modeling [88-90].

To characterize the rules of the regulatory network between JNK/AP-1 and JAK/STAT that ultimately drives emergence of spatial segregation to organize tissue stress responses, we developed a mathematical model using partial differential equations. These described the spatio-temporal evolution of JNK/AP-1 and JAK/STAT activation depending on relative concentrations of components in the system (Fig. 3A-C) [86, 91]. We defined a set of experimentally determined rules, namely: (1) JNK/AP-1 effectors propagate JNK/AP-1 via production of paracrine factors, such as *egr* or diffusible ROS [28, 54, 92]; (2) JNK/AP-1 effectors activate JAK/STAT via production of paracrine factors, such as *upd*'s [20, 28, 49, 51]; (3) Both pathways use positive feedback loops to enhance and stabilize their own activation [42, 55, 56, 93] and (4) JAK/STAT represses JNK/AP-1 cell-autonomously, via the ZEB-1

homologue Zfh2 (Fig. S3A-C) [51]. To simplify the model (Fig. 3B), we excluded negative feedback loops on JNK/AP-1 and JAK/STAT [94-96], as they often have a more modulatory function in noise buffering, temporal processing or saturation dynamics [97].

As we found experimentally that JNK/AP-1 repressed JAK/STAT cell-autonomously, we also designed a mutual repression model that, together with the aforementioned rules, included a rule that JNK/AP-1 represses JAK/STAT signaling (Fig. 3A,B') [98]. Using an unbiased sampling approach of a large parameter space, we compared the ability of the two models to produce simple bistable patterns (Fig. 3D). We found that the mutual repression model produced 140-fold more positive outcomes than a uni-directional model (10588 vs. 72 solutions) (Fig. 3E). When we switched from scoring simple bistable patterns (Fig. 3D) to observed bistable patterns (Fig. 3D') of JNK/AP-1 and JAK/STAT signaling in *egr*-expressing wing discs, the difference in competencies between the two models became even more prominent (Fig. 3E'). The uni-directional model was not sufficient to recapitulate observed bistable patterns, which suggests that the rule of JNK/AP-1 cell-autonomously repressing JAK/STAT is necessary for generating the segregated JNK/AP-1 and JAK/STAT signaling patterns seen during tissue stress. Accordingly, we show that a mutual-repression network lies at the core of JNK/AP-1 and JAK/STAT signaling interactions in response to tissue damage. Supporting this result, mutual repression networks have been extensively described to guide the formation of bistable states [99-105]. Furthermore, these networks can be further enhanced by self-activation loops, such as those integrated in our model [98, 102, 106]. Indeed, if JNK/AP-1 and JAK/STAT cross-talk were to completely lack mutual repression, JNK/AP-1 and JAK/STAT signaling would be expected to expand from the wound site in an unrestrained manner. Thus, the mutual repression regulatory network also ensures that these signaling pathways remain restricted during regeneration.

The length scale-independent space utilized by the model raises the possibility that this mutual repression network could act at length scales ranging from neighboring cells to multicellular tissues. *In vivo*, length scales may depend on the level of network activation and thus the concentration of non-autonomously acting cytokines (Egr, Upd's), as suggested by experiments shown in (Fig. 2K-P and Fig. S1.2). Indeed, we observed that the number of segregating solutions produced by the model increased with increasing rates of JNK/AP-1 activity (Fig. S3D). This observation parallels the progressive segregation of signaling domains found experimentally upon chronic activation of JNK/AP-1 signaling (Fig. 2K-P).

Taken together we propose that JNK/AP-1 initiates a mutual repression network between JNK/AP-1 and JAK/STAT signaling that, via the production of Upd's, facilitates the spatial

268 segregation of these two pathways. The spatial segregation of JNK/AP-1 and JAK/STAT via
269 mutual repression allows the tissue to maintain a JNK-dependent G2 stall in the presence of
270 pro-mitogenic signals, and restricts proliferation to a separate cell population. Thus, induction
271 of JNK/AP-1 signaling upon tissue damage and mutual repression with JAK/STAT is sufficient
272 to set up temporal and spatial signaling patterns, and functionally distinct cell populations.
273

274 **Figure 4**
275 **Ptp61F represses JAK/STAT activity in JNK/AP-1 signaling cells**
276

277 To understand how JNK/AP-1 may cell-autonomously repress JAK/STAT activation, we first
278 asked if JNK/AP-1 downregulates core components of the JAK/STAT pathway. Our previous
279 study demonstrated that the core components *dome*, *hop* and *stat92E* [107] were not
280 transcriptionally altered in *egr*-expressing discs [51]. Supporting this observation, we found
281 that the expression of GFP-tagged Hop [108] and Stat92E proteins from functionally validated
282 lines were unaltered in *egr*-expressing cells (Fig. S4.1A-I). This suggested that repression of
283 JAK/STAT signaling was likely due to altered function of regulators of JAK/STAT signaling. We
284 thus analyzed known negative regulators in *egr*-expressing discs. Levels of dPIAS/Su(var)2-
285 10 [109] and Apontic [110] were unchanged, but *ken* expression [111] was elevated in *egr*-
286 expressing discs (Fig. S4.2A-F). However, neither knock-down of *ken* nor *dPIAS/Su(var)2-10*
287 increased JAK/STAT activity in *egr*-expressing cells (Fig. S4.2G-L). Similarly, we could not
288 reproduce findings that knockdown of *apontic* reinstated JAK/STAT signaling in *egr*-expressing
289 discs (Fig. S4.2M-O) [112].
290

291 We then turned to the tyrosine phosphatase *Ptp61F,* a known repressor of Stat92E activation
292 [113, 114] which is transcriptionally induced in JNK-dependent tumors and other stress
293 conditions [23, 115]. Repression of JAK/STAT signaling by Ptp61F may occur at the level of
294 receptor complex signaling or Stat92E activity [113, 114, 116]. We found that knock-down of
295 *ptp61F* caused upregulation of the Stat92E reporter activity in high JNK-signaling cells of *egr*-
296 expressing discs (Fig. 4A-E, Fig. S4.3A-C). This suggests that Ptp61F is specifically required
297 in JNK-signaling cells to suppress Stat92E activity. Hence, to test if dephosphorylation of
298 Stat92E in JNK-signaling cells is rate-limiting for JAK/STAT signaling, we overexpressed an
299 HA-tagged Stat92E [117] in *egr*-expressing cells to saturate the dephosphorylation capacity of
300 Ptp61F. Indeed, *egr,stat92E-HA*-coexpressing cells in the central pouch domain activated the
301 JAK/STAT reporter at higher levels when compared to *egr*-expressing discs (Fig. 4F-J, Fig.
302 S4.3D,E). In contrast, co-expression of the JAK *hop* failed to induce JAK/STAT reporter activity
303 in *egr*-expressing discs (Fig. S4.3F-I). Importantly, this demonstrates that while signal
304 transduction from Upd ligands down to the effector Stat92E is in principle active in JNK-

305 signaling cells, it is the Ptp61F-mediated dephosphorylation of Stat92E that becomes rate-
306 limiting for JAK/STAT signaling.
307

308 **Figure 5**
309 **JAK/STAT repression is required for the G2 stall and protects central wound cells from**
310 **apoptosis**
311

312 As spatial segregation of JNK/AP-1 and JAK/STAT signaling domains was a robust feature of
313 *egr*-expressing discs, we wondered if the mutual repression network was necessary for
314 regeneration. We therefore forced expression of Stat92E in high JNK/AP-1 signaling cells, and
315 closely monitored cell and tissue level responses, such as cell survival and proliferation. The
316 loss of JNK/AP-1 and JAK/STAT segregation led to a pronounced increase in apoptosis (Fig.
317 5A-D). Co-expression of UAS-GFP confirmed that these apoptotic cells originated from cells
318 where JNK/AP-1 and JAK/STAT were forcibly co-activated (Fig. S5A-D).
319

320 We thus hypothesized that JAK/STAT interfered with the protective G2-stall of high JNK-
321 signaling cells, and thereby increased apoptosis [31, 37], While Stat92E expression altered
322 the proportion of S-phase cells in undamaged control discs, we observed no changes in the
323 proportion of cells in G1 or G2 (Fig. S5E-H). In contrast, a cell cycle analysis of *egr,Stat92E-*
324 *HA* co-expressing domains revealed a substantial increase in G1 and S-phase cells, indicating
325 that JNK-signaling cells failed to stall in G2 (Fig. 5E-J). Thus, forced Stat92E activation in high
326 JNK-signaling cells overrides the protective JNK-induced G2 stall and consequently increases
327 apoptosis. Supporting these results, we found that knock-down of Ptp61F in *egr*-expressing
328 discs also led to an increase in cycling cells in the tissue (Fig. 5O,P) correlating with increased
329 levels of apoptosis (Fig. 5K-N). This highlights the necessity of JNK/AP-1 and JAK/STAT
330 signaling segregation within the tissue, as it maintains the G2 stalled cell population. Thus, the
331 JNK-induced mutual repression network ensures the establishment of two distinct and
332 indispensable cell populations - one that stalls in G2 to prevent apoptosis and secrete
333 necessary pro-mitogenic factors like Upd and other paracrine effectors like Dilp8 and ImpL2
334 [20, 74], and a second that is able to respond to mitogenic signals and undergoes proliferation
335 necessary for regeneration. The strong similarities with paracrine signaling centers regulating
336 spatial patterning networks linked to specific cell behaviors and fate during development
337 encourages us to propose that the JNK-induced mutual repression network with JAK/STAT
338 functions as a central wound organizer network [118, 119].
339

340 **Figure 6**
341 **The wound organizer network segregates JNK/AP-1 and JAK/STAT signaling to drive**
342 **oncogenic growth**
343
344 Our observation that tissue repair processes after chronic damage support the establishment
345 of a signaling-sending and a responding cell population, which are respectively G2-stalled or
346 proliferative, led us to speculate that such a wound organizer network could well-support a
347 tumor microenvironment. Interestingly, the coexistence of JNK/AP-1 and JAK/STAT pathways
348 in tumors has been extensively described [18-23] and the existence of signal-sending G2-
349 stalled cells controlling non-autonomous proliferation has been demonstrated [31]. However,
350 their spatial patterns have not been well defined.
351
352 To understand if JNK/AP-1 and JAK/STAT also engage in a mutual repression network under
353 tumor conditions, we analyzed discs with a reduction in the well-characterized *scrib* tumor-
354 suppressor gene [120] or ectopic expression of oncogenic Ras^{V12} [121, 122]. To generate a
355 large genetically homogenous tissue, we used the pouch specific *rn-GAL4* driver to express
356 Ras^{V12} or *scrib-RNAi* in the entire wing pouch. A wing pouch expressing oncogenic Ras^{V12} for
357 44 h did not activate JNK/AP-1 or JAK/STAT reporters, demonstrating that Ras^{V12} alone does
358 not co-opt either pathway (Fig. 6A,B, Fig. S6A,B). RNAi-driven knock-down of *scrib* induced
359 barrier dysfunction (Fig. S6C,D) and, consistent with previous reports [123, 124], moderately
360 activated both JNK/AP-1 and JAK/STAT in the pouch (Fig. S6E-H). A closer examination,
361 however, revealed that the *scrib-RNAi* expressing pouch predominantly segregated JNK/AP-
362 1 and JAK/STAT signaling at the level of small cell clusters, while only few cell clusters
363 displayed co-activation of both pathways (Fig. 6C). This pattern mirrored our observations of
364 short-term *egr*-expression (Fig. S2L,M), thus suggesting that different contexts of mild JNK-
365 activation cause segregation at short distances. These observations indicate that JNK/AP-1
366 activation upon barrier dysfunction via disruption of cell polarity also induces the wound
367 organizer network, which subsequently sets up a bistable segregation of both pathways even
368 at a short range between neighboring cells.
369
370 Curiously, if Ras^{V12} and *scrib* mutations occur in the same cell, they cooperate to cause
371 dramatic overproliferation of imaginal discs, which is not observed in single Ras^{V12} or *scrib*
372 mutant tissues. This cooperativity is thought to be driven by activation of JNK/AP-1, as a
373 consequence of *scrib* disrupting cell polarity. JNK/AP-1 signaling then induces *upd's* and
374 JAK/STAT activation, which can be utilized by a Ras^{V12} controlled network to drive overgrowth
375 [18, 122]. We thus wondered if Ras^{V12} may disable the mutual repression motif between
376 JNK/AP-1 and JAK/STAT. This would allow autocrine activation of JAK/STAT by JNK signaling
377 in the same cell, as generally described in the literature [18, 122]. Cells driven to cycle despite

activation of JNK/AP-1 may then be protected by anti-apoptotic functions of Ras^{V12}. We wanted to test this hypothesis and closely monitored JNK/AP-1 and JAK/STAT signaling as well as cell proliferation in Ras^{V12} and *scrib RNAi*-coexpressing discs. Surprisingly, we found that JNK/AP-1 and JAK/STAT activation were still clearly segregated into exclusive signaling domains (Fig. 6D-F, Fig. S6I) with proliferation still predominantly associated with JAK/STAT (Fig. 6G-I). This suggests that even in a Ras^{V12} and *scrib* cooperativity model, where all cells are genetically identical, bistability driven by the mutual repression network organizes the pouch into distinct JNK/AP-1 or JAK/STAT-signaling domains. This observation aligns with previous observations that cooperativity between Ras and *scrib* can rely, in principle, on non-autonomous interactions [18, 125]. Crucially however, our observations demonstrate that non-autonomous cooperation between JNK/AP-1-activating and Ras^{V12} mutation arises as a self-organizing principle from a wound organizer network creating segregating signaling domains in a genetically homogenous tumorigenic tissue. Moreover, it suggests that activation of this wound organizer network and segregation of signaling and proliferative tasks may provide an advantage to tumors over autocrine integration of JNK/AP-1 and JAK/STAT signaling in the same cell.

Discussion

Previous studies established a role for JNK/AP-1 in promoting regeneration upon injury [35, 59]. This role is, in part, mediated by the expression of *upd's* which are essential to promote proliferation and survival via JAK/STAT signaling in a non-autonomous manner [28, 35, 48, 51, 126-129]. By identifying a mutual repression network between JNK/AP-1 and JAK/STAT, we conceptually establish JNK as a core organizer of tissue repair with parallels to organizers of cell fate patterning in developing tissues. The rules of the wound regulatory network we describe have important implications. Previous reports suggest that JNK/AP-1 activates itself non-autonomously, for example via activation of paracrine *egr* or ROS [28, 54, 92]. This implies that JNK/AP-1 activation could result in unchecked spatial expansion of JNK/AP-1-signaling, and accordingly of JAK/STAT signaling. Their unrestrained co-expansion would likely result in chronic inflammation. However, the mutual repression motif restrains the expansion of both JNK/AP-1 and JAK/STAT signaling domains, thus defining stable regions of inflammatory (mitogenic signals) and regenerative (pro-proliferative) responses at the start of the tissue repair process. We demonstrate that molecular mediators of mutual repression are Zfh2 downstream of JAK/STAT [51], and Ptp61F downstream of JNK.

At the level of cell behaviors, JNK/AP-1 and JAK/STAT generate mutually exclusive responses (Fig. 7B). JNK/AP-1 signaling supports wound center behaviors like tissue sealing [24, 25, 78],

415 cell fusion [14], establishment of paracrine signaling and importantly, an apoptosis-resistant
416 state that depends on a G2 cell cycle stall [31]. In contrast, JAK/STAT signaling supports
417 wound-distal behaviors, such as compensatory proliferation, potentially via upregulation of G1-
418 S cyclins such as CycD or CycE [130, 131].
419
420 By reducing Stat92E activation upon damage, JNK/AP-1 creates a permissive state for the G2
421 stall by preventing cycling, thereby protecting cells from apoptosis in a cytotoxic, inflammatory
422 environment (Fig. 7C; Fig. S1A). When high levels of both pathways are co-activated in the
423 same cell, the anti-apoptotic state of the G2 stall is in conflict with proliferative outcomes and
424 therefore active cycling which causes an increase in apoptosis. Consequently, apoptosis of
425 these wound center signaling cells could jeopardize wound organizer function and ultimately
426 tissue repair behaviors (Fig.7D). Therefore, the JNK-activated G2-stall and JAK/STAT-
427 activated cell cycling need to be spatially segregated. Importantly, our observations indicate
428 that, with exceptions [132], even successful tumors avoid coexistence of JNK and JAK/STAT
429 signaling in the same cell as they do not profit from the conflicting cell-autonomous inputs on
430 proliferation and survival decisions. Instead, tumors may highjack a wound organizer network
431 to chronically activate paracrine mitogenic signals but outsource proliferation to other cells as
432 a consequence of the senescent properties of JNK-signaling, G2-stalled cells [31].
433
434 We find that spatial patterns generated by the JNK/AP-1 and JAK/STAT regulatory network
435 act at different length scales, depending on the duration and strength of JNK/AP-1 activation
436 (Fig. 7A). Transient or low JNK/AP-1 activity generates short range spatial segregation of
437 JNK/AP-1 and JAK/STAT. Specifically, we observed their segregation between neighboring
438 cells upon transient expression of *egr* or mild disruption of barrier function in *scrib*-RNAi
439 expressing tissue. Cell-by-cell segregation may allow tissue repair processes to respond
440 dynamically. Switching between (1) a stalled, apoptosis-resistant state supporting survival and
441 paracrine signaling in an inflammatory environment or (2) a regenerative, proliferative
442 phenotype could facilitate continuous integration of the repair state. As wound healing
443 progresses and the JNK/AP-1 signaling field shrinks, shifting spatial probabilities of stalled and
444 cycling cells would ultimately resolve the damage.
445
446 In contrast, prolonged or high JNK/AP-1 activity segregates JAK/STAT signaling more robustly
447 and consequently into larger bistable fields, prolonging wound resolution and increasing the
448 risk for chronic wound pathologies. Indeed, prolonged JNK/AP-1 activity in Ras^{v12},*scrib*-RNAi
449 tumors or by chronic expression of *egr* drives robust bistable segregation of JNK/AP-1 and
450 JAK/STAT signaling into larger domains. Similarly, chronic wounds in human patients display
451 striking segregation of cellular responses. In a central inflammatory domain, cells acquire

452 senescent phenotypes, arrest and fail to proliferate. In the periphery, a hyperproliferative edge
453 is evidence of chronic mitogenic signals emanating from the wound [3, 9, 10, 133]. These
454 clinical patterns resemble patterning in *egr*-expressing discs, thereby suggesting that
455 *Drosophila* imaginal discs may represent a suitable model to study wound pathologies driven
456 by prolonged activation of inflammatory signaling.

457 **Materials and Methods**
458

459 **Fly stocks**

460 All fly stocks and experimental crosses were maintained on standard media and raised at 18 °C
461 or room temperature (22 °C) unless otherwise specified. For detailed genotypes, please refer
462 to Table S1 and S2.
463

464 Table S1 Key Resources Table

Designation - Genetic reagent (*D. melanogaster*)	**Source or Reference**	**Identifiers**	**Additional information**
'RFP': Act5C>FRT.CD2>GAL4, UAS-RFP	PMID: 29494583	BDSC: 30558	
'apt-RNAi': UAS-apt RNAi (TRiP.JF02134)	PMID: 26320097	BDSC: 26236	
'Dif-GFP': Pbac(Dif-GFP.FPTB)VK00033		BDSC: 42673	
dome^{G0441}	PMID: 11751581	BDSC: 12030	
'dome-GFP': FlyFos (dome::TGVBF)	PMID: 26896675	VDRC: 318098	
'E2F1-FUCCI, CycB-FUCCI'; Ubi-GFP.E2f1$^{1-230}$, Ubi-mRFP1.NLS.CycB$^{1-266}$	PMID: 24726363	BDSC: 55123	
'egr^{ts}': tub-GAL80ts, UAS-egr			I.Hariharan, University of California, Berkeley
'en>RFP': enGAL4, UAS-mRFP.NLS		BDSC: 30557	
GFP-HP-1	PMID: 18316477	BDSC: 30561	
gstD-GFP	PMID: 18194654		D. Bohmann, University of Rochester Medical Center
'hep^{act}': UAS-hep^{act}	PMID: 10903185	BDSC: 9306	
'hop': UAS-hop.H	PMID: 7796812	BDSC: 79033	
hop^{34}	PMID: 3095163		N. Perrimon
'hop-GFP': FlyFos (hop::TGVBF)	PMID: 26896675	VDRC: 318158	
hsflp^{122}			D. Bilder, University of California, Berkeley
'GFP': UAS-GFP S56T		BDSC: 1521	
'ken-LacZ': P{PZ}ken^{02970}	PMID: 10471706	BDSC: 11244	
'ken-RNAi': UAS-ken-RNAi (TRiP.HMS01219)	PMID: 26320097		
'ptp61f-RNAi': UAS-ptp61f-RNAi $^{i2-5}$	PMID: 19398577	BDSC: 56510	
'p35': UAS-p35.H	PMID: 9144202	BDSC: 5072	
'rn(ts)>': rn$^{GAL4\text{-}DeltaS}$, tubGAL80^{ts}		BDSC: 8142	recombinant
'rn(ts)>egr': rn$^{GAL4\text{-}5}$, UAS-egr, tubP-GAL80^{ts}		BDSC: 8142; BDSC: 7018	recombinant; I.Hariharan, University of California, Berkeley
'Ras^{v12}, scrib-RNAi': UAS-Ras^{v12},scrib-RNAi			M. Uhlirova, University of Cologne
'Ras^{v12}': UAS-Ras^{v12}			H. Richardson, University of Melbourne
'rnE/F-EGFP': rn(E/F)-EGFP (ds Red+)	PMID: 26497147		P. Volkan, Duke University School of Medicine
'scrib-RNAi': UAS-scrib RNAi			M. Uhlirova, University of Cologne
'Stat92E': UAS-Stat92E.ORF.3xHA	PMID: 23583758	Fly-ORF F000750	
'Stat92E^{85c9} ': FRT82B stat92E^{85c9}			E. Bach, New York University School of Medicine
'Stat92E>dGFP': 10xStat92E>dGFP	PMID: 17008134		

'Stat92E>GFP': 10xStat92E>GFP	PMID: 17008134		
'Stat92E-GFP': PBac{Stat92E-GFP.FLAG}^{VK00037}		BDSC: 38670	
'su(var)2-10-GFP': PBac{Su(var)2-10-GFP.FPTB}^{VK00037}	PMID: 29284660	BDSC: 64795	
'su(var)2-10-RNAi': UAS- Su(var)2-10-RNAi $^{TRiP.HMS00750}$	PMID: 26320097	BDSC: 32956	
'TRE>RFP': TRE-DsRed.T4	PMID: 22509270		
'Xbp1-GFP': UAS-Xbp1-GFP.HG	PMID: 23160805	BDSC: 60731	
upd3.1-3-LacZ			I.Hariharan, University of California, Berkeley
upd-LacZ: upd>LacZ (PD1)	PMID: 8582614		Y. H. Sun, Academia Sinica, Taipei
w^{118}			
'zfh2': UAS-Zfh2[EAB]	PMID: 24172131		F. Diaz-Benjumea, Universidad Autónoma de Madrid

Antibodies / Commercial Kits	**Source or Reference**	**Identifiers**	**Dilution**
Chicken anti-GFP	Abcam	Cat. #: ab13970	(1:1000)
Mouse anti-H3-pS10	Abcam	Cat. #: ab14955	(1:2000)
Mouse anti-β-Galactosidase	Promega	Cat. #: Z3782	(1:1000)
Mouse monoclonal anti-MMP-1	DSHB	Cat. #: 3A6B4	(1:30)
Mouse monoclonal anti-MMP-1	DSHB	Cat. #: 3B8D12	(1:30)
Mouse monoclonal anti-MMP-1	DSHB	Cat. #: 5H7B11	(1:30)
Mouse monoclonal anti-Nubbin	DSHB	Cat. #: 2D4	(1:100)
Mouse monoclonal anti-α-Tubulin	Sigma-Aldrich	Cat. #: T9026	(1:5000)
Rabbit anti-cleaved Dcp-1	Cell Signaling	Cat. #: 9578	(1:200)
Rabbit monoclonal anti-GFP	Invitrogen	Cat. #: G10362	(1:200)
Rabbit polyclonal anti-apt	R. Schuh, MPI-MS, Göttingen	NA	(1:200)
Rat monoclonal anti-GFP	Chromotek	Cat. #: 3H9-100	(1:1000)
Rat monoclonal anti-HA	Monoclonal Antibody Core Facillity at the Helmholtz Zentrum München	Clone #: 3F10	(1:20)
Rat monoclonal anti-RFP		Clone #: 5F8	(1:20)
Click-iT Plus EdU Alexa Fluor 647 Imaging Kit	Invitrogen	Cat. #: C10640	

PMID – PubMed Identifier; BDSC – Bloomington Drosophila Stock Center; DSHB – Developmental Studies Hybridoma Bank, VDRC – Vienna Drosophila Stock Collection, FlyORF – University of Zurich ORFeome Project

465

466 Table S2. Detailed Genotypes

Figure	**Panel**	**Genotype**	**Temperature shift**	**Recovery**
Figure 1	A	*TRE-RFP; 10xStat92E>dGFP*	24h 30°C, D7 AED	-
Figure 1	B	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure 1	C	*Ubi-GFP.E2f1$^{1-230}$, Ubi-mRFP1.NLS.CycB$^{1-266}$; rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure 1	D	*Ubi-GFP.E2f1$^{1-230}$, Ubi-mRFP1.NLS.CycB$^{1-266}$; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure 1	E	*TRE-RFP; Upd3.1-3-LacZ/rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure 1	F,M	*TRE-RFP; Upd3.1-3-LacZ/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure 1	G,I,K	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure 1	H,J,L,N	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S1.1	D	*rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-

Figure S1.1	E	*rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S1.1	F	*Upd-LacZ/X; TRE-RFP; TM6B, tub-GAL80*	24h 30°C, D7 AED	-
Figure S1.1	G	*Upd-LacZ/X; TRE-RFP; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S1.1	H	*SuVar2-10 GFP/rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure S1.1	I	*SuVar2-10 GFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S1.1	J	*gstD-GFP; rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure S1.1	K	*gstD-GFP; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S1.1	L	*UAS-Xbp1-GFP.HG/ rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure S1.1	M	*UAS-Xbp1-GFP.HG/ rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S1.1	N	*TRE-RFP; Dif-GFP/rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure S1.1	O	*TRE-RFP; Dif-GFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S1.2	B,E	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	0h 30°C, D7 AED	-
Figure S1.2	C,F	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	7h 30°C, D7 AED	-
Figure S1.2	D,G	*TRE-RFP; 10xStat92E>dGFP/TM6B, tub-GAL80^{ts}*	14h 30°C, D7 AED	-
Figure S1.2	H,K	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	0h 30°C, D7 AED	-
Figure S1.2	I,L	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	7h 30°C, D7 AED	-
Figure S1.2	J,M	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	14h 30°C, D7 AED	-
Figure 2	A	*hsflp^{122}; UAS-p35; 10xStat92E>dGFP/Act5C.GAL4 (FRT.CD2), UAS-RFP*	10' HS 37°C, D7 AED	28h 22°C
Figure 2	B,C	*hsflp^{122}; UAS-p35/UAS-hep^{act}; 10xStat92E>dGFP/Act5C.GAL4 (FRT.CD2), UAS-RFP*	10' HS 37°C, D7 AED	28h 22°C
Figure 2	D	*hsflp^{122}; UAS-p35/UAS-hep^{act}; 10xStat92E>dGFP/Act5C.GAL4 (FRT.CD2), UAS-RFP*	7' HS 37°C, D5 AED	48h 22°C
Figure 2	E	*TRE-RFP; 10xStat92E>dGFP*	24h 30°C, D7 AED	-
Figure 2	F	*enGAL4-mRFP.NLS/TRE-RFP; 10xStat92E>dGFP/tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure 2	G	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	7h 30°C, D7 AED	-
Figure 2	H,K,N	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	7h 30°C, D7 AED	-
Figure 2	I	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	14h 30°C, D7 AED	-
Figure 2	J,L,O	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	14h 30°C, D7 AED	-
Figure 2	M,P	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S2	A	*hsflp^{122}; UAS-p35; 10xStat92E>dGFP/Act5C.GAL4 (FRT.CD2), UAS-RFP*	10' HS 37°C, D7 AED	28h 22°C
Figure S2	B	*hsflp^{122}; UAS-p35; 10xStat92E>dGFP/Act5C.GAL4 (FRT.CD2), UAS-RFP*	7' HS 37°C, D7 AED	28h 22°C
Figure S2	C	*hsflp^{122}; UAS-p35/UAS-hep^{act}; 10xStat92E>dGFP/Act5C.GAL4 (FRT.CD2), UAS-RFP*	7' HS 37°C, D7 AED	28h 22°C
Figure S2	D	*hsflp^{122}; UAS-p35; rn(E/F)-EGFP/Act5C.GAL4 (FRT.CD2), UAS-RFP*	7' HS 37°C, D7 AED	28h 22°C
Figure S2	E,F,G	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	0h,7h,14h 30°C, D7 AED	-
Figure S2	H,I,J,K	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	0h,7h,14h 30°C, D7 AED	-
Figure S2	L	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	7h 30°C, D7 AED	-
Figure S2	M	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	7h 30°C, D7 AED	-
Figure S3	A, C	*rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S3	B, C	*rn-GAL4, tub-GAL80^{ts}, UAS-egr; UAS-zfh2^{EAB}-mCherry*	24h 30°C, D7 AED	-

Figure 4	A	*10xStat92E>dGFP/UAS-ptp61F RNAi^{i2-5}; rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure 4	B,D,E	*10xStat92E>dGFP; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure 4	C,D,E	*10xStat92E>dGFP/UAS-ptp61F RNAi $^{i2-5}$; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure 4	F,J	*CycE-lacZ/10xStat92E>dGFP; rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure 4	G,J	*CycE-lacZ/10xStat92E>dGFP; UAS-Stat92E-3xHA/rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure 4	H,J	*CycE-lacZ/10xStat92E>dGFP; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure 4	I,J	*CycE-lacZ/10xStat92E>dGFP; UAS-Stat92E-3xHA/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S4.1	A	*dome^{G0441};; | dome^{G0441};;dome-GFP*	24h 30°C, D7 AED	-
Figure S4.1	B	*hop^{34};; | hop^{34};;hop-GFP*	24h 30°C, D7 AED	-
Figure S4.1	C	*stat^{85c9}/TM6c | stat^{85c9}/stat^{85c9} | Stat92E-GFP; stat^{85c9}/TM6c | Stat92E-GFP; stat^{85c9}/stat^{85c9}*	24h 30°C, D7 AED	-
Figure S4.1	D	*Stat92E-GFP | GFP-HP1 | w$^{[118]}$*	D7 AED	-
Figure S4.1	E	*w$^{[118]}$*	24h 30°C, D7 AED	-
Figure S4.1	F	*hop-GFP/TM6B, tub-GAL80*	24h 30°C, D7 AED	-
Figure S4.1	G	*hop-GFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S4.1	H,J,M	*Stat92E-GFP;*	24h 30°C, D7 AED	-
Figure S4.1	I	*Stat92E-GFP; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S4.1	K,M	*UAS-p35/Stat92E-GFP; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S4.2	A	*Su(var)2-10-GFP; rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure S4.2	B	*Su(var)2-10-GFP; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S4.2	C	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure S4.2	D	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S4.2	E	*ken-LacZ^{02970};*	24h 30°C, D7 AED	-
Figure S4.2	F	*ken-LacZ^{02970}; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S4.2	G	*10xStat92E>dGFP/UAS-ken-RNAi; rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure S4.2	H,K	*10xStat92E>dGFP; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S4.2	I	*10xStat92E>dGFP/UAS-ken-RNAi; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S4.2	J	*10xStat92E>dGFP; UAS-Su(var)2-10-RNAi/rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure S4.2	L	*10xStat92E>dGFP; UAS-Su(var)2-10-RNAi/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S4.2	M	*10xStat92E>dGFP; UAS-apt-RNAi/rn-GAL4, tub-GAL80^{ts}*	48h 30°C, D5 AED	-
Figure S4.2	N	*10xStat92E>dGFP; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	48h 30°C, D5 AED	-
Figure S4.2	O	*10xStat92E>dGFP; UAS-apt-RNAi/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	48h 30°C, D5 AED	-
Figure S4.3	A,C	*CyO; 10xStat92E>dGFP*	D7 AED	-
Figure S4.3	B,C	*enGAL4, UAS-mRFP.NLS/UAS-ptp61F RNAi; 10xStat92E>dGFP*	D7 AED	-
Figure S4.3	D	*UAS-GFP; rn-GAL4, tub-GAL80^{ts}*	12h 30°C, D7 AED	-
Figure S4.3	E	*UAS-GFP; UAS-Stat92E-3xHA/rn-GAL4, tub-GAL80^{ts}*	12h 30°C, D7 AED	-
Figure S4.3	F	*10xStat92E>dGFP; rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure S4.3	G	*10xStat92E>dGFP/UAS-hop.H; rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-

Figure S4.3	H	*10xStat92E>dGFP; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S4.3	I	*10xStat92E>dGFP/UAS-hop.H; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure 5	A,C,D	*TRE-RFP; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure 5	B,C ,D	*TRE-RFP; UAS-Stat92E-3xHA/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure 5	E,G,H	*Ubi-GFP.E2f1$^{1-230}$, Ubi-mRFP1.NLS.CycB$^{1-266}$; rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure 5	F,G,H	*Ubi-GFP.E2f1$^{1-230}$, Ubi-mRFP1.NLS.CycB$^{1-266}$; UAS-Stat92E-3xHA/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure 5	I	*UAS-GFP; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure 5	J	*UAS-Stat92E-3xHA/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure 5	K,O,M,N	*UAS-GFP; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure 5	L,P,M ,N	*UAS-GFP/UAS-ptp61F RNAi^{i2-5}; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S5	A	*UAS-GFP; rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure S5	B	*UAS-GFP; UAS-Stat92E-3xHA/rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure S5	C	*UAS-GFP; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S5	D	*UAS-GFP; UAS-Stat92E-3xHA/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S5	E	*Ubi-GFP.E2f1$^{1-230}$, Ubi-mRFP1.NLS.CycB$^{1-266}$; rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure S5	F	*Ubi-GFP.E2f1$^{1-230}$, Ubi-mRFP1.NLS.CycB$^{1-266}$; rn-GAL4, tub-GAL80^{ts}, UAS-egr*	24h 30°C, D7 AED	-
Figure S5	G	*UAS-GFP; rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure S5	H	*UAS-Stat92E-3xHA/rn-GAL4, tub-GAL80^{ts}*	24h 30°C, D7 AED	-
Figure 6	A	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	44h 30°C, D6 AED	-
Figure 6	B	*UAS-Ras^{v12}/TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	44h 30°C, D6 AED	-
Figure 6	C,C',G	*UAS-scrib-RNAi/TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	44h 30°C, D6 AED	-
Figure 6	D,D',E,F,H,I	*UAS-Ras^{v12},scrib-RNAi/TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	44h 30°C, D6 AED	-
Figure S6.1	A,E	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	44h 30°C, D6 AED	-
Figure S6.1	B	*UAS-Ras^{v12}/TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	44h 30°C, D6 AED	-
Figure S6.1	C	*10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	48h 30°C, D6 AED	-
Figure S6.1	D	*UAS-scrib-RNAi; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	48h 30°C, D6 AED	-
Figure S6.1	F	*UAS-scrib-RNAi/TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	44h 30°C, D6 AED	-
Figure S6.1	G,I	*UAS-Ras^{v12},scrib-RNAi/TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}*	44h 30°C, D6 AED	-
Figure S6.1	H	*TRE-RFP; 10xStat92E>dGFP/rn-GAL4, tub-GAL80^{ts}, UAS-egr*	44h 30°C, D6 AED	-

467

468

469

470

471 **Fly genetics**

472 Fosmids carrying GFP-tagged *hop* (VDRC 318158) and *Stat92E* (BDSC 38670) alleles,
473 expressed proteins at expected molecular weights and, at least partially complemented
474 respective null alleles, suggesting that both GFP-fusion proteins are functional. A potential
475 Dome-GFP fosmid (VDRC 318098) failed to give rise to a GFP protein by immunofluorescence
476 and Western blot analysis and failed to complement a *dome* allele, thus Dome levels could not
477 directly be analyzed. Genetic complementation assays were carried out by crossing the GFP-
478 tagged fosmids into the background of LOF mutants ($dome^{G0441}$, hop^{9p5} and $stat^{85C9}$). For X-
479 linked $dome^{G0441}$ and hop^{9p5} alleles, we calculated the percentage of hemizygous viable adult
480 males emerging from control and experimental crosses (Fig. S4.1A,B). For the autosomal
481 $stat^{85C9}$ allele, we determined pupariation success of homozygous mutant larvae by counting
482 the number of pupariated larvae in control and experimental crosses (Fig. S4.1C).

483

484 We validated RNAi lines by demonstrating that they caused changes to the developmental
485 pattern of JAK/STAT activity in the hinge if expressed throughout normal wing disc
486 development. Crosses were set as previously described, using the *en-GAL4* driver to express
487 the RNAi in the posterior compartment. Larvae were kept at 18°C till D7 AEL. On D7, early L3
488 staged larvae were dissected, showing characteristic JAK/STAT reporter activity pattern in the
489 hinge. Following dissection and staining, sibling controls and RNAi expressing discs were
490 mounted on the same slide. All discs were imaged at the same settings and the JAK/STAT
491 reporter intensities were quantified in selected regions in the anterior and posterior hinge (Fig.
492 S4.3C). Intensity values from the anterior compartment (not expressing the RNAi) was used
493 as the internal control.

494

495 **Flip-out clones**

496 GAL4/UAS-driven 'flip-out' overexpression experiments utilized heat-shock-driven expression
497 of a flippase to clonally express *a UAS* construct of choice. After 6h of egg collections, heat-
498 shock was induced on developmental day 5 or 6 at 37°C for 7-10 min . Larvae were dissected
499 at wandering 3rd instar stage or as indicated (28h or 48h after heat-shock, Fig. 2A-D, S2.A-
500 D).

501

502 **Genetic cell ablation using Gal4/UAS/Gal80ts**

503 To induce expression of *egr*, experiments were carried out as described in [31, 38, 51] with
504 few modifications. Briefly, larvae of genotype *rn-GAL4, tub-GAL80*^{ts} and carrying the desired
505 *UAS*-transgenes were staged with a 6h egg collection and raised at 18°C at a density of 50
506 larvae/vial. Overexpression of transgenes was induced by shifting the temperature to 30°C for
507 24h at D7 after egg deposition (AED), and larvae dissected at recovery time point R0 h (Fig.

508 S1.1C) unless noted otherwise. For time course and tumor experiments, transgenes were
509 induced by shifting the temperature to 30°C for 0h, 7h, 14h or 24h at D7 (Fig. S1.2, 2 and S2),
510 or 44h at D6 (Fig. 6 and S6) respectively. Larvae were subsequently dissected for analysis or
511 allowed to recover at 22°C for the indicated time. All images represent R0 h unless noted
512 otherwise. Control genotypes were either *rn*^{ts}>, or sibling animals (*+/TM6B, tubGAL80 or*
513 *+/TM6c*) (Smith-Bolton et al. 2009). All experiments were performed with ≥ 2 biological
514 replicates.
515
516 **Immunohistochemistry**
517 Wing discs from third instar larvae were dissected and fixed for 15 min at room temperature in
518 4% paraformaldehyde in PBS. Washing steps were performed in PBS containing 0.1%
519 TritonX-100 (PBT). Discs were then incubated with primary antibodies (described in **Table S1)**
520 in PBT, gently mixing overnight at 4°C. Tissues were counterstained with DAPI (0.25 ng/µl,
521 Sigma, D9542), Phalloidin-Alexa Fluor 488/647 (1:100, Life Technologies) or Phalloidin-
522 conjugated TRITC (1:400, Sigma) during incubation with cross-absorbed secondary antibodies
523 coupled to Alexa Fluorophores (Invitrogen or Abcam) at room temperature for 2h. Tissues were
524 mounted using SlowFade Gold Antifade (Invitrogen, S36936). Whenever possible,
525 experimental and control discs were processed in the same vial and mounted on the same
526 slides to ensure comparability in staining between different genotypes. Images were acquired
527 using the Leica TCS SP8 Microscope, using the same confocal settings and processed using
528 tools in Fiji. Of note, all wing discs expressing the *10xStat92E>dGFP* reporter were boosted
529 with an anti-GFP antibody.
530
531 **EdU Labelling**
532 EdU incorporation was performed using the Click-iT Plus EdU Alexa Fluor 647 Imaging Kit,
533 (described in **Table S1)** prior to primary antibody incubation. Briefly, larval cuticles were
534 inverted in Schneider's medium and incubated with EdU (10µM final concentration) at RT for
535 15 minutes. Cuticles were then fixed in 4% PFA/PBS for 15 minutes, washed for 30 minutes
536 in PBT 0.5%. EdU-Click-iT labeling was performed according to manufacturer's guidelines.
537 Tissues were washed in PBT 0.1%, after which immunostainings, sample processing and
538 imaging were carried out as described above.
539
540 **Western Blots**
541 Cell lysates from third instar wing imaginal discs and brains were prepared in lysis buffer (50
542 mM Tris-HCl pH 7.5, 300mM NaCl, 0.1mM EDTA, 1% Triton X-100, 0.1% SDS, 5% Glycerol,
543 1mM PMSF, 1/10 tablet of Complete Mini Protease Inhibitor Cocktail) on ice. Protein samples
544 were loaded onto a 10% polyacrylamide gel, along with Chameleon Duo Pre-stained Protein

545 Ladder (LI-COR, P/N 928-60000) as a molecular weight ladder and run at 150V. After SDS-
546 PAGE, proteins were transferred onto a nitrocellulose membrane (Bio-Rad, 162-0115,
547 0.45µm) in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) using the wet-
548 tank method with a current density of 300mA for 1h. Prior to antibody incubation, membranes
549 were blocked with 5% milk in PBS. Membrane was incubated with primary antibodies in PBS-T
550 (1% Triton-X in 1xPBS) - rat anti-GFP (Chromotek, 3H9-100, 1:1000) and mouse anti-α-
551 tubulin (Sigma, T9026, 1:5000) on a rotative plate at 4°C overnight. Primary antibodies were
552 removed and washed in dH_20 twice for 5 mins each. Membrane was then incubated with
553 secondary antibodies diluted in PBS-T - donkey α-mouse secondary (LI-COR, 926-68072,
554 1:20000) labelled with a 700nm IRDye and goat α-rat (LI-COR, 926-32219, 1:20000) labelled
555 with an 800nm IRDye. Membrane was washed in dH_20 twice for 5 mins each before detection
556 using the Image Studio Software on the Odyssey SA system (LI-COR) (Fig. S4.1D).
557
558 **Image Analysis and Quantification**
559
560 **General comments**
561 Images were processed, analyzed and quantified using tools in Fiji (ImageJ v2.0.0) [134].
562 Extreme care was taken to apply consistent methods (i.e. number of projected sections,
563 thresholding methods, processing) for image analysis. Figure panels were assembled using
564 Affinity Designer v1.10. Statistical analyses were performed in Graphpad Prism or R v3.3.3
565 (www.R-project.org).
566
567 **Total disc area quantification**
568 Maximum intensity projections of z-stacks for each disc were obtained in Fiji. Masks were
569 generated after applying a fixed threshold (10-255) to the DAPI channel and noise was reduced
570 using the 'Despeckle' function. Area measurement from the resulting masks were obtained
571 using the 'Measure' function.
572
573 ***10xStat92E>dGFP* quantification**
574 Maximum intensity projections of selected confocal sections were taken, excluding the
575 peripodium and carefully chosen to capture signaling activity within the disc proper. In the *egr*-
576 expressing or *egr,transgene*-coexpressing discs, a mask of the pouch domain was generated.
577 Region Of Interest (ROI) outlines were either manually drawn on the DAPI channel to include
578 the pouch and hinge domains till the medial hinge fold or by thresholding Nubbin staining.
579 Centroid co-ordinates were identified using the 'Measure' function and marked with a point
580 tool. To measure the fluorescence intensity of the *Stat92E>dGFP* reporter, a square ROI

581 (25x25μm) was placed centrally over the centroid and mean intensity within the ROI was
582 obtained using the 'Measure' function in Fiji. (Fig. 4)
583
584 **Dcp-1 area quantification**
585 Maximum intensity projections of stacks for each disc were obtained in Fiji. Masks were
586 obtained by thresholding for signal of interest. Signal to noise ratio was enhanced by 'Remove
587 outliers' (bright, radius = 1.5) or 'Despeckle' functions. The '3D object counter' function was
588 used to obtain surface area of the generated mask. To control for differences arising from the
589 total size of the discs, ratios between the Dcp-1 area and total WID area were obtained for
590 each disc and reported as percentage values. (Fig. 5C,D,M,N)
591
592 **Tracing JNK, JAK/STAT, EdU and Upd reporters profiles**
593 Partial max projections from confocal stacks of the disc proper were generated, carefully
594 excluding any peripodial signal. For hinge profiles of the JAK/STAT reporter, size-matched
595 discs were chosen and line traces were manually drawn along the hinge using the polygon
596 selection tool. Reporter intensity values from the 'Plot profile' function were averaged for 0h,
597 7h and 14h control and *egr*-expressing discs and plotted. (Fig. S1.2)
598 For reporter patterns along disc center to disc periphery, JNK/AP-1 masks were made using
599 thresholding tools and ROI outlines were generated. Based on ROIs, the centroid value was
600 determined and $n \geq 15$ tracks were drawn from the centroid outwards towards the hinge for a
601 fixed distance - covering the JNK/AP-1 domain, the JNK/AP-1-JAK/STAT interface, and the
602 JAK/STAT signaling domain respectively. Using the 'Plot profile' function in Fiji, the
603 fluorescence intensity values of each reporter was obtained along these tracks and averaged.
604 Reporter patterns for each disc was graphed by scaling the averaged values from each track,
605 between 0 to 1 and plotted on the Y-axis. This was done independently for n≥3 discs, and a
606 representative image was selected for visualization of the spatial trends of the reporters. (Fig
607 1M,N)
608
609 **Density plots for JNK/AP-1 and JAK/STAT reporters**
610 For combined pouch and hinge regions (Fig. 1I,J), the ROI outlines were manually drawn on
611 the DAPI channel to include the pouch and hinge domains till the medial hinge fold. Partial
612 max projections of z-sections from the disc proper were generated, carefully excluding any
613 peripodial signal.
614
615 For time course experiments with *egr*-expressing discs (S2.H-K), a central z-section was
616 chosen and ROIs generated by thresholding the *TRE-RFP* channel to include only the JNK-
617 signaling pouch domain. After applying a 'Gaussian blur' filter (sigma=2), masks were

618 generated by applying a low fixed threshold (value = 10) based on early, 7h *egr*-expressing
619 discs. Noise removal was done using the 'Despeckle' function, followed by 'Fill Hole' function.
620 ROI outlines were generated using 'Create selection'. All ROIs were placed on the source
621 *TRE>RFP* and *Stat92E>dGFP* reporter channels. 'Save X-Y co-ordinates' function was used
622 to obtain pixel fluorescence intensity values for both reporters within the ROI, and the
623 distribution of fluorescence intensities across the samples were graphed by generating a
624 binned (bins = 16) 2D histogram plot using R. After all NA values were set to 0, data density
625 per bin for each disc was calculated as a percentage. Values were averaged across
626 corresponding bins for all discs within a timepoint to generate the average fluorescence
627 intensity distribution profile. To distinguish regions with low, medium and high signaling for
628 each pathway, the graphs were subdivided into sections s1-s9 by defining a threshold to
629 distinguish low, medium and high JNK/AP-1 and JAK/STAT signal intensities (Fig. S2K).
630 Percentage of datapoints within each section was determined from the averaged fluorescence
631 intensity distribution profile. Ordinary 2-way ANOVA with Dunnett's multiple comparisons test
632 was carried out for statistical analysis. Alpha was set at 0.05
633
634 **Cell cycle profiles**
635 Representative sections were selected per disc and a square ROI area (75x75μm^2) was placed
636 over the central pouch cells. Only viable cells stained with characteristic euchromatic and
637 heterochromatic DAPI staining were chosen. Using the 'Multi point' tool, cells showing a G1
638 profile (green), G2 profile (yellow+red) or neither (black) were counted. Percentage values for
639 the gap phases were calculated for n≥3 discs per genotype and visualized. T-tests were
640 performed on the calculated G1:G2 ratios per disc. (Fig 5G,H)
641
642 **JAK/STAT reporter quantifications for testing RNAi lines**
643 Crosses were setup as previously described and wing discs from larvae were dissected on D7
644 AED. Single sections at similar focal planes were carefully chosen during imaging. Using
645 drawing tools in Fiji, ROIs were generated to include JAK/STAT signaling cells in the hinge
646 within A and P compartments. The mean JAK/STAT reporter intensity within the ROI was
647 obtained from control and RNAi expressing discs using the 'Measure' function in Fiji. For each
648 dataset, paired t-tests were performed between A and P fluorescence intensity values for the
649 same genotype. (Fig. S4.3A-C)
650
651 **Nuclear translocation of Stat92E-GFP**
652 Images from the pouch (n=12) and hinge (n=9) domains of control discs and pouch (n=16) and
653 hinge (n=12) domains of *p35+egr* expressing discs were obtained to track the intra-cellular
654 localization of *Stat92E-GFP* within the nucleus and cytoplasm. Images were taken at high

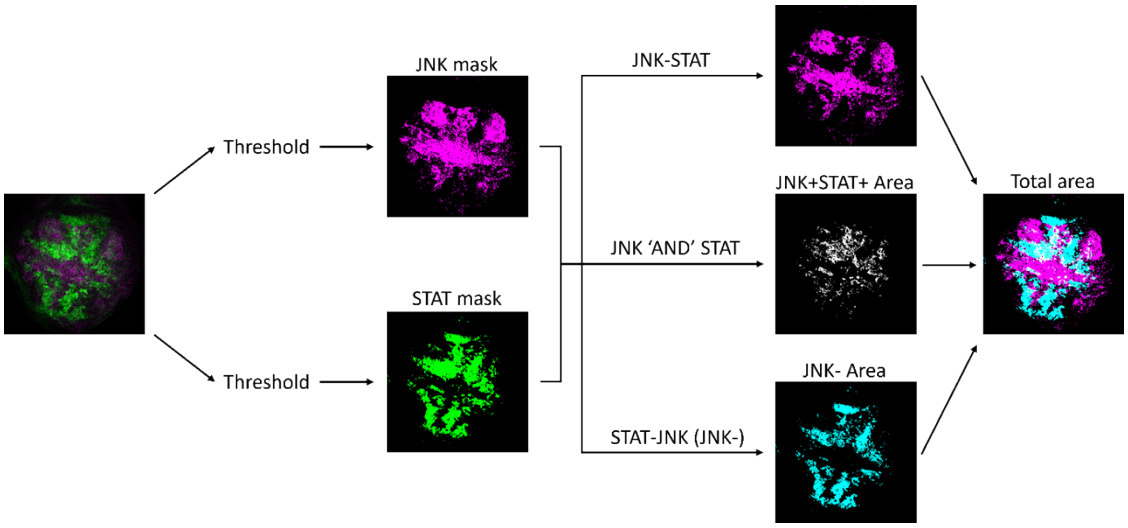
655 magnification (63x). After thresholding ('Huang'), the selection tool was used to generate a
656 nuclear ROI using the DAPI channel. The inverse selection tool was used to generate a
657 corresponding cytoplasmic ROI for each image (Fig S4.1L). These nuclear and cytoplamic
658 ROIs were then placed on the *Stat92E-GFP* channel and mean fluorescence intensity values
659 were obtained for each subcellular fraction and unpaired t-tests were performed. (Fig. S4.1M)
660

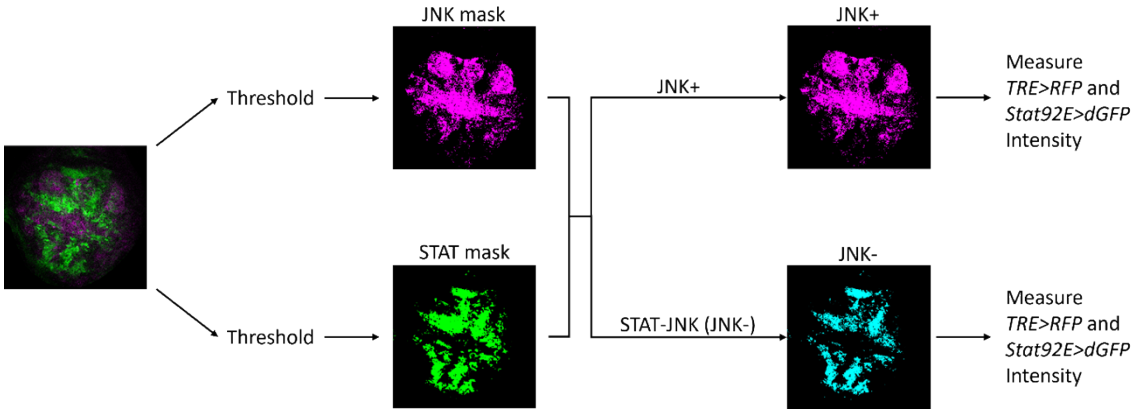
661 **MMP-1 intensity quantification**

662 To measure the fluorescence intensity of MMP-1 in *egr*-expressing and *egr,zfh2-co*expressing
663 discs, maximum intensity projections of confocal sections were taken. Sections were carefully
664 chosen to capture JNK/AP-1 signaling activity in the disc proper, avoiding the peripodium and
665 cell debris. Square ROIs of fixed length (30 µm) were placed in 3 non-overlapping regions in
666 the pouch domain, and mean intensity was calculated using the 'Measure' function in Fiji. Mean
667 fluorescence intensities obtained from ROIs were averaged per disc and values from each disc
668 per genotype were used for statistical analysis. (Fig. S3A-C)
669

670 **JNK$^+$ and JNK$^-$ Area and intensity quantifications (*Ras^{v12},scrib-RNAi* tumors)**

671 A representative z-section was selected per disc. As our interest was in quantitating the spatial
672 segregation, only JNK/AP-1 or JAK/STAT signaling regions in the tumor were chosen. After
673 applying a 'Gaussian blur' filter (sigma=2), 'Moments' thresholding and 'Despeckle' function
674 for noise correction was applied to generate masks for regions showing *TRE>RFP* (JNK) and
675 *Stat92E>dGFP* (JAK/STAT) reporter activity. From the 'Image calculator' function, 'AND' and
676 'Subtract' operations on JNK/AP-1 and STAT masks were carried out to obtain the JNK$^+$STAT$^+$
677 mask, and the exclusive JNK$^+$ (JNK-STAT) or exclusive JNK$^-$(STAT-JNK) masks respectively.
678 Area of each mask was obtained using the 'Measure' function. Summed area of these masks
679 were considered as the total area and each area was then represented as a percentage of
680 total. One-way paired ANOVA with Holm-Šídák's multiple comparison test was performed to
681 test for statistical significance. Mean intensity for *TRE>RFP* or *10xStat92E>dGFP* reporters
682 were measured inside the JNK/AP-1 mask (JNK$^+$) and the exclusive JNK$^-$ masks. Paired t-
683 tests were performed to test for statistical significance. (Fig. 6E,F and S6I)
684





ph3 counts

Partial maximum intensity projection of three central z-sections were taken per disc. To generate masks for regions showing *Stat92E>dGFP* reporter activity ($STAT^+$), a 'Gaussian blur' filter (sigma=1), 'Moments' threshold and 'Despeckle' function for noise correction was applied. The DAPI channel was used to manually outline the pouch domain and generate masks. Using the 'Image calculator' function, $STAT^+$ mask was 'Subtract'-ed from the pouch mask to generate the $STAT^-$ mask. Areas of the $STAT^+$ and $STAT^-$ regions in the pouch were obtained using the 'Measure' function. Then, the ph3 channel was duplicated and 'Li' threshold was applied. 'Remove outlier' (radius=5 threshold=50) and 'Despeckle' function for noise correction, followed by 'Watershed' function was applied to generate a ph3 mask. Using the 'AND' operator, the ph3 mask was overlayed with the $STAT^+$ and $STAT^-$ masks. '3D object counter' function was then used to obtain ph3 'Object counts' within the $STAT^+$ and $STAT^-$ regions and divided by area of each ROI to get the ph3 counts per mm^2. Paired t-tests were performed to test for statistical significance. (Fig. 6I)

Mathematical modelling

To test for the existence of a regulatory motif within our signaling network, a mathematical model was derived to describe the temporal dynamics of the concentration of specific molecules over a fixed 2D space. The system was modeled as a set of ordinary differential equations (ODEs) (see also Fig. 3C). Two sets of modeling equations were established, that reflected conditions which include or exclude repression of JAK/STAT by JNK. The term describing repression of JAK/STAT (referred to as 'JAK' in the modeling equations and hereafter) by JNK/AP-1 is highlighted in red.

Uni-directional repression model (see also Fig. 3B):

$$
\begin{aligned}
\frac{d\mathrm{EIG}(t,x)}{dt} &= b_{EIG}(x) - k_{deg,EIG}\mathrm{EIG} + k_{act,EIG}\frac{\mathrm{JNK}^{n_1}}{K^{n_1}_{m,act,EIG} + \mathrm{JNK}^{n_1}} + D_{EIG}\nabla^2 EIG \\
\frac{d\mathrm{JNK}(t,x)}{dt} &= b_{JNK} - k_{deg,JNK}\mathrm{JNK} + k_{act,JNK}\frac{\mathrm{EIG}^{n_2}}{(K^{n_2}_{m,act,JNK} + \mathrm{EIG}^{n_2})(1 + k^{ni1}_{inh,JNK,by,JAK}\mathrm{JAK}^{ni1})} \\
\frac{d\mathrm{JAK}(t,x)}{dt} &= b_{JAK} - k_{deg,JAK}\mathrm{JAK} + k_{act,JAK}\mathrm{UPD}\frac{\mathrm{JAK}^{n_3}}{(K^{n_3}_{m,act,JAK} + \mathrm{JAK}^{n_3})} \\
\frac{d\mathrm{UPD}(t,x)}{dt} &= b_{UPD} - k_{deg,UPD}\mathrm{UPD} + k_{act,UPD}\frac{\mathrm{JNK}^{n_4}}{K^{n_4}_{m,act,UPD} + \mathrm{JNK}^{n_4}} + D_{UPD}\nabla^2 UPD
\end{aligned}
$$

Mutual repression model (see also Fig. 3B'):

$$
\begin{aligned}
\frac{d\mathrm{EIG}(t,x)}{dt} &= b_{EIG}(x) - k_{deg,EIG}\mathrm{EIG} + k_{act,EIG}\frac{\mathrm{JNK}^{n_1}}{K^{n_1}_{m,act,EIG} + \mathrm{JNK}^{n_1}} + D_{EIG}\nabla^2 EIG \\
\frac{d\mathrm{JNK}(t,x)}{dt} &= b_{JNK} - k_{deg,JNK}\mathrm{JNK} + k_{act,JNK}\frac{\mathrm{EIG}^{n_2}}{(K^{n_2}_{m,act,JNK} + \mathrm{EIG}^{n_2})(1 + k^{ni1}_{inh,JNK,by,JAK}\mathrm{JAK}^{ni1})} \\
\frac{d\mathrm{JAK}(t,x)}{dt} &= b_{JAK} - k_{deg,JAK}\mathrm{JAK} + k_{act,JAK}\mathrm{UPD}\frac{\mathrm{JAK}^{n_3}}{(K^{n_3}_{m,act,JAK} + \mathrm{JAK}^{n_3})(1 + k^{ni2}_{inh,JAK,by,JNK}\mathrm{JNK}^{ni2})} \\
\frac{d\mathrm{UPD}(t,x)}{dt} &= b_{UPD} - k_{deg,UPD}\mathrm{UPD} + k_{act,UPD}\frac{\mathrm{JNK}^{n_4}}{K^{n_4}_{m,act,UPD} + \mathrm{JNK}^{n_4}} + D_{UPD}\nabla^2 UPD
\end{aligned}
$$

Characteristic units are represented by state (u), space (x) and time (t). The mathematical terms include, among others, basal rate of production (*b*), activation rate (k_{act}), linear degradation rate (k_{deg}), Michaelis–Menten constant (K_m), inhibition constant (k_{inh}), diffusion co-efficients (D_{EIG}, D_{UPD}), Hill co-efficients to model cooperativity (n_1,n_2,n_3,n_4, ni_1,ni_2) and Hill kinetics to describe self-amplification and mutual repression.

The first equation describes the dynamics of the concentration of Egr, produced at a basal rate dependent on the spatial co-ordinate x and degraded at a linear rate. Hill kinetics describe the production of Egr induced by JNK/AP-1 and its diffusion away from the source. The second equation describes the dynamics of the concentration of the JNK/AP-1 TF, produced at a basal rate and degraded linearly. Hill kinetics describe production of JNK/AP-1 induced by *egr* as well as inhibition of JNK/AP-1 by JAK [51]. The third equation describes the dynamics of the

concentration of the JAK TF, produced at a basal rate and degraded linearly. Hill kinetics describe positive feedback dependent on Upd. Inhibition of JAK by JNK/AP-1 is described and the model is tested with and without this mathematical term (highlighted in red) to check if this interaction exists within the system. The fourth equation describes the dynamics of the concentration of Upd, produced at a basal rate and degraded linearly. Hill kinetics describe the production of Upd induced by JNK/AP-1 and its diffusion [28, 48, 51].

Technically, the modeling was done based on a non-dimensionalized version of the model, rescaled to reduce the number of free parameters. The system was then defined as follows:

$$
\begin{aligned}
\frac{d\mathrm{EIG}(t,x)}{dt} &= \gamma\left(1(x) - \mathrm{EIG} + k_{act,EIG}\,\frac{\mathrm{JNK}^{n_1}}{K^{n_1}_{m,act,EIG} + \mathrm{JNK}^{n_1}}\right) + \nabla^2 EIG \\
\frac{d\mathrm{JNK}(t,x)}{dt} &= \gamma\left(1 - k_{deg,JNK}\mathrm{JNK} + k_{act,JNK}\,\frac{\mathrm{EIG}^{n_2}}{(K^{n_2}_{m,act,JNK} + \mathrm{EIG}^{n_2})(1 + k^{ni1}_{inh,JNK,by,JAK}\mathrm{JAK}^{ni1})}\right) \\
\frac{d\mathrm{JAK}(t,x)}{dt} &= \gamma\left(1 - k_{deg,JAK}\mathrm{JAK} + k_{act,JAK}\mathrm{UPD}\,\frac{\mathrm{JAK}^{n_3}}{(K^{n_3}_{m,act,JAK} + \mathrm{JAK}^{n_3})(1 + k^{ni2}_{inh,JAK,by,JNK}\mathrm{JNK}^{ni2})}\right) \\
\frac{d\mathrm{UPD}(t,x)}{dt} &= \gamma\left(1 - k_{deg,UPD}\mathrm{UPD} + k_{act,UPD}\,\frac{\mathrm{JNK}^{n_4}}{K^{n_4}_{m,act,UPD} + \mathrm{JNK}^{n_4}}\right) + d\nabla^2 UPD
\end{aligned}
$$

For each dimensionless parameter, the parameter dependencies and the scanned parameter range are represented in the table below:

Dimensionless parameter	Functional relation to systems parameter	Scanned parameter range
γ	$k_{deg,EIG}\ L^2\ /\ D_{EIG}$	$0.01 \ldots 10000$
$\hat{k}_{deg,JNK}$	$k_{deg,JNK}\ /\ k_{deg,EIG}$	$0.01 \ldots 10000$
$\hat{k}_{deg,JAK}$	$k_{deg,JAK}\ /\ k_{deg,EIG}$	$0.01 \ldots 10000$
$\hat{k}_{deg,Upd}$	$k_{deg,Upd}\ /\ k_{deg,EIG}$	$0.01 \ldots 10000$
$\hat{k}_{act,EIG}$	$k_{act,EIG}\ /\ b_{EIG}$	$0.00001 \ldots 10$
$\hat{k}_{act,JNK}$	$k_{act,JNK}\ /\ b_{JNK}$	$0.01 \ldots 10000$
$\hat{k}_{act,JAK}$	$k_{act,JAK}\ D_{EIG}\ /\ L^2\ /\ b_{UPD}\ /\ b_{JAK}$	$0.01 \ldots 10000$
$\hat{k}_{act,UPD}$	$k_{act,UPD}\ /\ b_{UPD}$	$0.01 \ldots 10000$
$\hat{K}_{m,act,EIG}$	$K_{m,act,EIG}\ D_{EIG}\ /\ L^2\ /\ b_{EIG}$	$0.00001 \ldots 10$
$\hat{K}_{m,act,JNK}$	$K_{m,act,JNK}\ D_{EIG}\ /\ L^2\ /\ b_{JNK}$	$0.01 \ldots 10000$
$\hat{K}_{m,act,JAK}$	$K_{m,act,JAK}\ D_{EIG}\ /\ L^2\ /\ b_{JAK}$	$0.01 \ldots 10000$
$\hat{K}_{m,act,UPD}$	$K_{m,act,UPD}\ D_{EIG}\ /\ L^2\ /\ b_{UPD}$	$0.01 \ldots 10000$
$\hat{k}_{inh,JAK,by,JNK}$	$k_{inh,JAK,by,JNK}\ b_{JAK}\ L^2\ /\ D_{EIG}$	$0.01 \ldots 10000$
$\hat{k}_{inh,JNK,by,JAK}$	$k_{inh,JNK,by,JAK}\ b_{JNK}\ L^2\ /\ D_{EIG}$	$0.01 \ldots 10000$
$\hat{n}_i$	n_i	$1 \ldots 4$
d	$D_{UPD}\ /\ D_{EIG}$	$1.5 \ldots 4$

We performed a global analysis by sampling approach introduced in *Rausenberger et. al* [135]. The models were simulated on a 1D spatial domain x = [0 1]. To represent the wound site, a high basal production of Egr was fixed within a finite space at x = [0 0.05], while basal rate of production of Egr for the rest of the region was set to zero. The rate of production of Upd was defined as a function of JNK/AP-1 activity [18, 20, 39, 51]. For all states, initial condition was set as u (t=0,x) = 0.1. Solutions for each model (with and without repression) were simulated using 10^6 parameter sets, drawn uniformly from an interval from 10^{-3} to 10^{+2} on a logarithmic scale, and tested under different conditions of diffusion – $D_{Upd} > D_{Egr}$, $D_{Upd} < D_{Egr}$ and $D_{Upd} = D_{Egr}$. The resulting solutions were automatically scored for countergradient endpoint features extracted from the experimentally observed gradient patterns.

Using this approach [135], positive solutions for a generic bistable pattern from the scanned parameter range were automatically extracted based on the following features:

i. JAK at x = 0 lower than JAK at x = 1
ii. JNK/AP-1 at x = 0 higher than JNK/AP-1 at x = 1
iii. Relative difference of JAK higher than 10%
iv. Relative difference of JNK/AP-1 higher than 10%

Positive solutions for a specific bistable pattern which mimics biologically observed patterns were automatically extracted based on the additional features:
v. JAK has a local maximum which is not at the boundary of the simulated domain
vi. Height of local JAK maximum at least 5%

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1244
1245

1246 **Figure 1**

1247 **JNK/AP-1 and JAK/STAT are activated in distinct spatial domains upon tissue damage**

1248

1249 **(A-B)** A control wing disc **(A)** and wing disc after 24h of *egr*-expression **(B)** in the pouch (R0) (see also **S1.1, B**)
1250 under the control of the inducible *rn-GAL4* (*rotund-GAL4*) driver (rn^{ts}>) in the pouch domain. Discs express the
1251 JNK/AP-1 reporter *TRE>RFP* and were stained for cleaved Dcp-1 to visualize apoptosis.

1252

1253 **(C-D)** A control wing disc **(C)** and wing disc after 24h of *egr*-expression **(D)** in the pouch (R0), under the control of
1254 the inducible *rn-GAL4* (*rotund-GAL4*) driver (rn^{ts}>). Discs also express the FUCCI cell cycle reporters *mRFP-NLS-*
1255 $CycB^{1-266}$ (red) and $GFP\text{-}E2f1^{1-230}$ (green) (see also **S1.1A**) and were assessed for DNA replication by EdU
1256 incorporation assay.

1257

1258 **(E-F)** A control **(E)** and *egr*-expressing disc **(F)** at R0, expressing the *upd3.1-3-LacZ* (gray) and the JNK/AP-1
1259 reporter *TRE>RFP* (magenta). Discs were stained for β-Galactosidase.

1260

1261 **(G-H)** A control **(G)** and *egr*-expressing disc **(F)** at R0, expressing the JNK/AP-1 reporter *TRE>RFP* (magenta) and
1262 the dynamic JAK/STAT reporter *Stat92E>dGFP* (green).

1263

1264 **(I-J)** 2D density plots mapping pixel fluorescence intensity values for the *TRE>RFP* (JNK intensity, X-axis) and
1265 *Stat92E>dGFP* (JAK/STAT intensity, Y-axis) reporters, measured in the combined wing pouch and hinge domains
1266 (black dashed lines in **O**) in control, *rn-GAL4*-expressing discs **(I)** or *egr*-expressing discs **(J)** . Dashed lines
1267 (cyan) represent visually defined thresholds distinguishing low or high reporter activity in the quadrants Q1,Q2,Q3
1268 and Q4. Note that less than 1% of pixels show both high JNK and high JAK/STAT signaling activity in the
1269 combined JNK and JAK/STAT signaling domains (i.e. pouch and hinge). Graphs represent pooled values from
1270 *n=4*, control and *n=3, egr*-expressing discs.

1271

1272 **(K-L)** A control **(K)** and *egr*-expressing disc **(L)** assessed for S-phase activity using EdU incorporation assays. Discs
1273 also express the *TRE>RFP* (magenta) and *Stat92E>dGFP* (green) reporters.

1274

1275 **(M-N)** Fluorescence intensity profiles for *upd3.1-3LacZ* (gray) and *TRE>RFP* (magenta) reporters **(M)**, or *TRE>RFP*
1276 (magenta), *Stat92E>dGFP* (green) reporters and EdU (yellow) reporter intensities **(N)** traced from the pouch center
1277 (PC) to the disc periphery (DP) (yellow arrows in **F** and **O**). Graph represents mean ± SEM of reporter fluorescence
1278 intensity values from *n=15* tracks across a representative disc, scaled to the maximum measured value.

1279

1280

1281 **(O)** Schematic representation of undamaged control discs and spatially segregated patterns of JNK/AP-1 (magenta)
1282 and JAK/STAT (green) reporter activity in 24h *egr*-expressing discs (R0).

1283

1284 Maximum projections of multiple confocal sections are shown in **K-L**.
1285 Discs were stained with DAPI to visualize nuclei. Scale bars: 50 μm

1286

1287

1288 **Figure 2**

1289 **JNK/AP-1 signaling represses JAK/STAT activity cell-autonomously**

1290

1291 **(A-C)** RFP expression marks *p35*-expressing control clones in the pouch (magenta, **A**) or *p35,hep^{act}*-expressing
1292 JNK+ clones in the pouch (magenta, **B**) and peripodium (magenta, **C**) 28h after clone induction in discs expressing
1293 the stable *Stat92E>GFP* reporter (green). Yellow arrowheads indicate clones of interest. Note the cell-autonomous
1294 repression of *Stat92E>GFP* reporter activity in JNK+ clones in the pouch periphery and hinge **(B)**, as well as in the
1295 peripodium **(C)**, when compared to control clones in the pouch **(A)** and peripodium **(**see **S2A)**

1296

1297 **(D)** *p35,hep^{act}*-expressing JNK+ clones in a disc expressing the *Stat92E>GFP* reporter (green), stained with anti-
1298 MMP-1 (magenta) - a downstream target of the JNK/AP-1 signaling pathway. The yellow circle highlights a JNK+
1299 clone of interest, which shows a strong cell-autonomous repression of the JAK/STAT reporter activity in the
1300 JAK/STAT-signaling competent hinge, 48h after clone induction.

1301

1302 **(E-F)** Control disc **(E)** and a disc expressing *egr* **(F)** in the posterior compartment using the *enGAL4* driver at R0.
1303 Dashed yellow line marks the A/P boundary. Both discs express the *Stat92E>dGFP* reporter (green) and are stained
1304 for the JNK/AP-1 target MMP-1 (magenta). White asterisk indicates JNK/AP-1 reporter activity in the posterior pouch
1305 region. Note the non-cell autonomous JAK/STAT reporter activity in the anterior pouch, indicated by yellow
1306 arrowheads in **F**.

1307

1308 **(G-J)** Control **(G,I)** and *egr*-expressing **(H,J)** discs at 7h or 14h after ablation expressing the *TRE>RFP* reporter
1309 (magenta) and assessed for S-phase activity using EdU incorporation assay (cyan). While the number of S-phase
1310 cells are uniformly distributed in the pouch domain of undamaged control discs **(G,I)**, note the successive decrease
1311 in the S-phase cells (cyan) from 7h **(H)** to 14h **(J)** of *egr*-expression, coinciding with increasing *TRE>RFP* reporter
1312 activity in the pouch. Insets show the same images, with settings adjusted to visualize the JNK/AP-1 activity in the
1313 pouch of 7h *egr*-expressing discs.

1314

1315 **(K-M)** A time-course analysis of *TRE>RFP* (magenta) and *Stat92E>dGFP* (green) reporter activity in *egr*-expressing
1316 discs after 7h **(K)**, 14h **(L)** and 24h **(M)** of ablation. Yellow squares show magnified regions. Yellow lines mark the
1317 interface between JNK/AP-1 and JAK/STAT signaling cells. Note the increasing dominance of JNK/AP-1 signaling
1318 and decreasing JAK/STAT activation inside the boundary of JNK/AP-1 signaling cells at the JNK/AP-1-JAK/STAT
1319 interface.

1320

1321 **(N-P)** Representative 2D density plots of pixel fluorescence intensity for the *TRE>RFP* (JNK intensity, X-axis) and
1322 *Stat92E>dGFP* (JAK/STAT intensity, Y-axis) reporters, measured exclusively within the JNK/AP-1 signaling pouch
1323 domain (magenta in Fig. **1O**) from 7h **(L)**, 14h **(M)** and 24h **(N)** *egr*-expressing discs. Black asterisks highlight the
1324 region of increase and subsequent decline in the density of co-localizing pixels with medium and high reporter
1325 activities from 7h to 14h **(N,O)**, and 14h to 24h **(O,P)** respectively.

1326

1327 Maximum projections of multiple confocal sections are shown in **E-J**.
1328 Discs were stained with DAPI to visualize nuclei. Scale bars: 50 μm

1329

1330

1331 **Figure 3**

1332 **A mutual-repression loop promotes bistable segregation of JNK/AP-1 and JAK/STAT activation patterns**

1333

1334 **(A-E')** Workflow to investigate the presence of a regulatory motif between the JNK/AP-1 and JAK/STAT signaling
1335 pathways driving pattern formation in *egr*-expressing discs. Fluorescence intensity profiles for the *TRE>RFP*
1336 (magenta) and *Stat92E>dGFP* (green) reporter patterns show bistable segregation in 24h egr-expressing discs.
1337 Intensity profiles are traced from the pouch center (PC) to the disc periphery (DP) **(A)**. Schematic showing the
1338 models tested – a uni-directional repression model acting only on JNK/AP-1 signaling **(B)** and a mutual repression
1339 model acting on both JNK/AP-1 and JAK/STAT signaling **(B')**. Modeling equations were generated, and a global
1340 analysis by sampling approach was used to analyze 21 selected parameters **(C)** (see also Fig.**S3**) and test for
1341 outcomes that produce bistable, switch-like patterns **(D-D')**. Number of simple bistable outcomes **(D)** from the
1342 mutual repression model (dark grey) are substantially greater than those from the uni-directional repression model
1343 (light grey) **(E)**. Applying stringent selection criteria based on the biologically observed reporter activity pattern **(D')**,
1344 positive outcomes reduce to 0 in the model without the mutual repression **(E')**.

1345

1346

Figure 4

Ptp61F represses JAK/STAT activity in JNK/AP-1 signaling cells

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(A-C) A *ptp61F-RNAi*-expressing **(A)**, *egr*-expressing **(B)** and *egr,ptp61F-RNAi*-co-expressing disc **(C)** at R0. All discs also express the *Stat92E>dGFP* reporter (green). Yellow circles highlight the central domain of remaining *rn-GAL4* expressing cells. Note the derepression and ectopic JAK/STAT activity in the *egr,ptp61F-RNAi*-co-expressing disc.

(D) Quantification of total area of wing discs. Graphs display mean ± SEM for *n=8*, *egr*-expressing and *n=10*, *egr,ptp61F-RNAi*-co-expressing discs. T-tests were performed to test for statistical significance.

(E) Quantification of the *Stat92E>dGFP* reporter fluorescence intensity measured within the JNK/AP-1 signaling pouch domain. Black square in schematic shows measured region. Graphs display mean ± SEM for *n=8*, *egr*-expressing and *n=10*, *egr,ptp61F-RNAi*-co-expressing discs. U-tests were performed to test for statistical significance.

(F-I) A control **(F)**, *stat92E*-expressing **(G)**, *egr*-expressing **(H)** and *egr,stat92E*-co-expressing disc **(I)**. All discs also express the dynamic *Stat92E>dGFP* reporter (green). Staining for HA confirms expression and nuclear/cytoplasmic localization of the *UAS-stat92E-3xHA* construct. Yellow arrowheads point to the restricted, non-autonomously induced JAK/STAT activity in the peripheral pouch in control discs **(F,G)**. Yellow circles highlight the central domain of remaining *rn-GAL4* expressing cells **(H,I)**.

(J) Quantification of the *Stat92E>dGFP* reporter fluorescence intensity measured within the JNK/AP-1 signaling pouch domain. Black square in schematic **(E)** shows measured region. Graphs display mean ± SEM for *n=16*, control discs (rn^{ts}>), *n=9*, *stat92E*-expressing discs, *n=11*, *egr*-expressing and *n=12*, *egr,stat92E*-co-expressing discs. One-way ANOVA with multiple comparisons was performed to test for statistical significance.

Discs were stained with DAPI (magenta) to visualize nuclei. Scale bars: 50 µm

Figure 5

JAK/STAT repression by JNK/AP-1 is required for the G2 arrest and protects central wound cells from apoptosis.

(A-B) An *egr*-expressing **(A)** and *egr,stat92E*-coexpressing disc **(B)** at R0, stained for DAPI (cyan), and cleaved Dcp-1 (red) to visualize apoptosis.

(C-D) Quantification of total wing disc area **(C)** and normalized area occupied by cleaved Dcp-1 **(D)**. Graphs display mean ± SEM for *n=10, egr*-expressing and *n=9, egr,stat92E*-co-expressing discs. T-tests were performed to test for statistical significance.

(E-F) An *egr*-expressing **(E)** and *egr,stat92E*-coexpressing disc **(F)** at R0, also expressing the FUCCI reporter. Dashed white squares highlight the distinct shift from G2 (yellow cells) to G1 (green cells) in the pouch domain.

(G-H) Bar graph **(G)** representing the cumulative proportion of gap phase cells in G1 (green) and G2 (yellow) for each genotype. Graph **(H)** displays G1-phase:G2-phase ratios obtained from *n=5, egr*-expressing and *n=3, egr,stat92E*-co-expressing discs. Dashed white squares in **E-F** show measured regions. T-test with Welch's correction was performed to test for statistical significance.

(I-J) EdU incorporation assay to detect S-phase cells (gray) in *egr*-expressing **(I)**, or *egr,stat92E*-co-expressing **(J)** disc. Discs were stained with DAPI (red) to visualize nuclei.

(K-L) An *egr*-expressing **(K)** and *egr,ptp61F-RNAi*-coexpressing **(L)** disc at R0, stained for DAPI (cyan), and cleaved Dcp-1 (red) to visualize apoptosis.

(M-N) Quantification of total wing disc area **(M)** and normalized area occupied by cleaved Dcp-1 **(N)**. Graphs display mean ± SEM for *n=11, egr*-expressing and *n=15, egr,ptp61F-RNAi*-coexpressing discs. T-tests were performed to test for statistical significance.

(O-P) EdU incorporation assay to detect S-phase cells (gray) in an *egr*-expressing **(O)**, and *egr,ptp61F-RNAi*-coexpressing **(P)** disc. Discs were also stained with DAPI (red) to visualize nuclei.

Maximum projections of multiple confocal sections are shown in **A-B, I-L, O-P**. Scale bars: 50 μm

1416 **Figure 6**

1417 **The wound organizer network segregates JNK/AP-1 and JAK/STAT signaling to drive oncogenic growth**

1418

1419 **(A-D)** A control wing disc **(A)** and wing discs expressing *Ras^{v12}* **(B)**, *scrib-RNAi* **(C, C')**, or *Ras^{v12},scrib-RNAi* **(D,D')**
1420 for 44 h D6 AED. Discs also express the JNK/AP-1 reporter *TRE>RFP* (magenta) and the JAK/STAT reporter
1421 *Stat92E>dGFP* (green). Yellow squares in mark area of magnification shown in **(C',D')**.

1422

1423 **(E)** Schematic shows the *TRE>RFP*-positive (JNK$^+$, magenta), *Stat92E>dGFP*-positive (STAT$^+$, green) and
1424 JNK/AP-1 and JAK/STAT double positive regions (JNK$^+$STAT$^+$, gray) selected for measurements. Graph represents
1425 normalized area of measured regions based on the total signaling area. Note that only 15% of regions within the
1426 tumor express both the JNK/AP-1 and JAK/STAT signaling reporters. Graph represents mean ± SEM for *n=6,*
1427 *Ras^{v12},scrib-RNAi*-expressing discs. One-way paired ANOVA with Holm-Šídák's multiple comparison test was
1428 performed to test for statistical significance.

1429

1430 **(F)** Schematic shows *TRE>RFP*-positive (JNK$^+$, magenta) and *TRE>RFP*-negative (JNK$^-$, white) regions selected
1431 for measurements. Graph represents *Stat92E>dGFP* fluorescence intensity measured within selected JNK$^+$ and
1432 JNK$^-$ regions. Note the reduction in the reporter intensity from 71.91±4.32 to 36.66±1.74 within the JNK$^-$ vs. JNK$^+$
1433 region. Graph represents mean ± SEM for *n=6,* ras^{v12},scrib-RNAi-expressing discs. Paired t-tests was performed
1434 to test for statistical significance.

1435

1436 **(G-H)** A wing disc expressing *scrib-RNAi* **(G)** and *Ras^{v12},scrib-RNAi* **(H)** at 44 h. Discs also express the
1437 *Stat92E>dGFP* reporter and were stained for phospho-Histone3 (ph3) to visualize cells in mitosis. Segmented
1438 masks of ph3 were generated for quantifications and an overlay of the *Stat92E>dGFP* reporter (green) and ph3
1439 (white) is represented. Orange dashed lines mark the STAT$^+$ regions within the discs.

1440

1441 **(I)** Schematic shows *Stat92E>dGFP*-positive (STAT$^+$, green) and *Stat92E>dGFP*-negative (STAT$^-$, white) regions
1442 selected for measurements. Graph represents ph3 counts normalized to area in the STAT$^+$ and STAT$^-$ regions for
1443 *n=5, Ras^{v12},scrib-RNAi*-expressing discs.

1444

1445 Maximum projections of multiple confocal sections are shown in **G-H**
1446 Discs were stained with DAPI to visualize nuclei. Scale bars: 50 µm

1447

1448

1449

1450 **Figure 7**

1451 **Mutual repression between JNK/AP-1 and JAK/STAT sets up wound organizer activity**

1452

1453 **(A-B)** Transient (7h *egr*-expression), and chronic (24h *egr*-expression or 44h *Ras^{v12},scrib-RNAi*-expression)
1454 activation of JNK/AP-1 signaling stratifies JNK-activating tissue into distinct JNK/AP-1 and JAK/STAT signaling
1455 domains **(A)** and cell behaviors **(B)**.

1456

1457 **(C)** *egr*-expressing discs spatially stratify tissue repair behaviors via a mutual repression motif. JNK/AP-1 represses
1458 JAK/STAT signaling activitiy via Ptp61F, while JAK/STAT represses JNK/AP-1 activation via Zfh2. JNK signaling
1459 ensures G2 arrest and cell survival, while JAK/STAT signaling ensures proliferation and supports survival in the
1460 absence of JNK.

1461

1462 **(D)** Disruption of the mutual repression motif by cell-autonomous activation of JNK/AP-1 and JAK/STAT signaling
1463 overrides the protective G2 stall and drives cells into mitotic cycling and G1, where p53 activation can lead to
1464 apoptotic outcomes.

1465

1466

1467

1468

Figure 1.

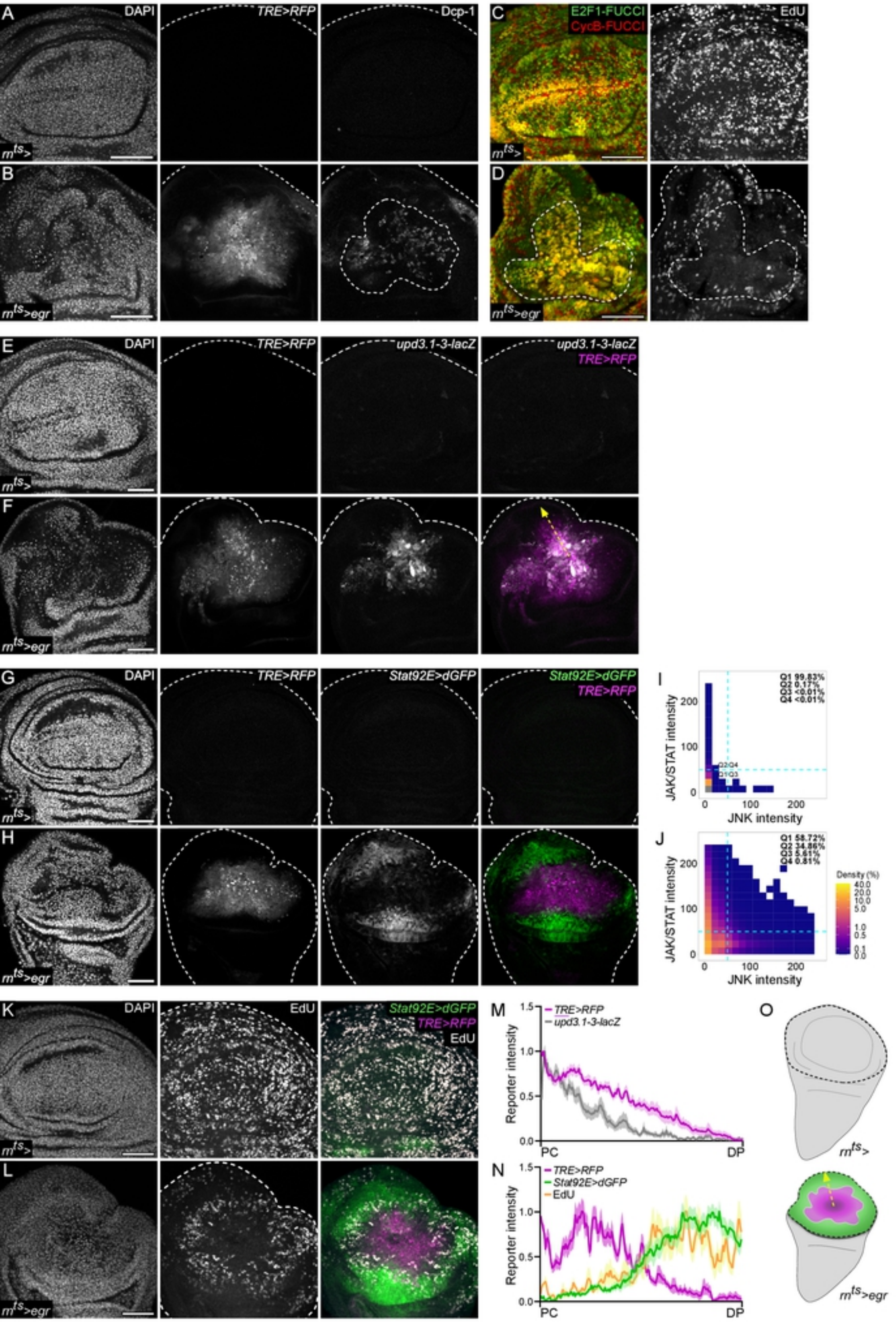

Figure 1

Figure 2

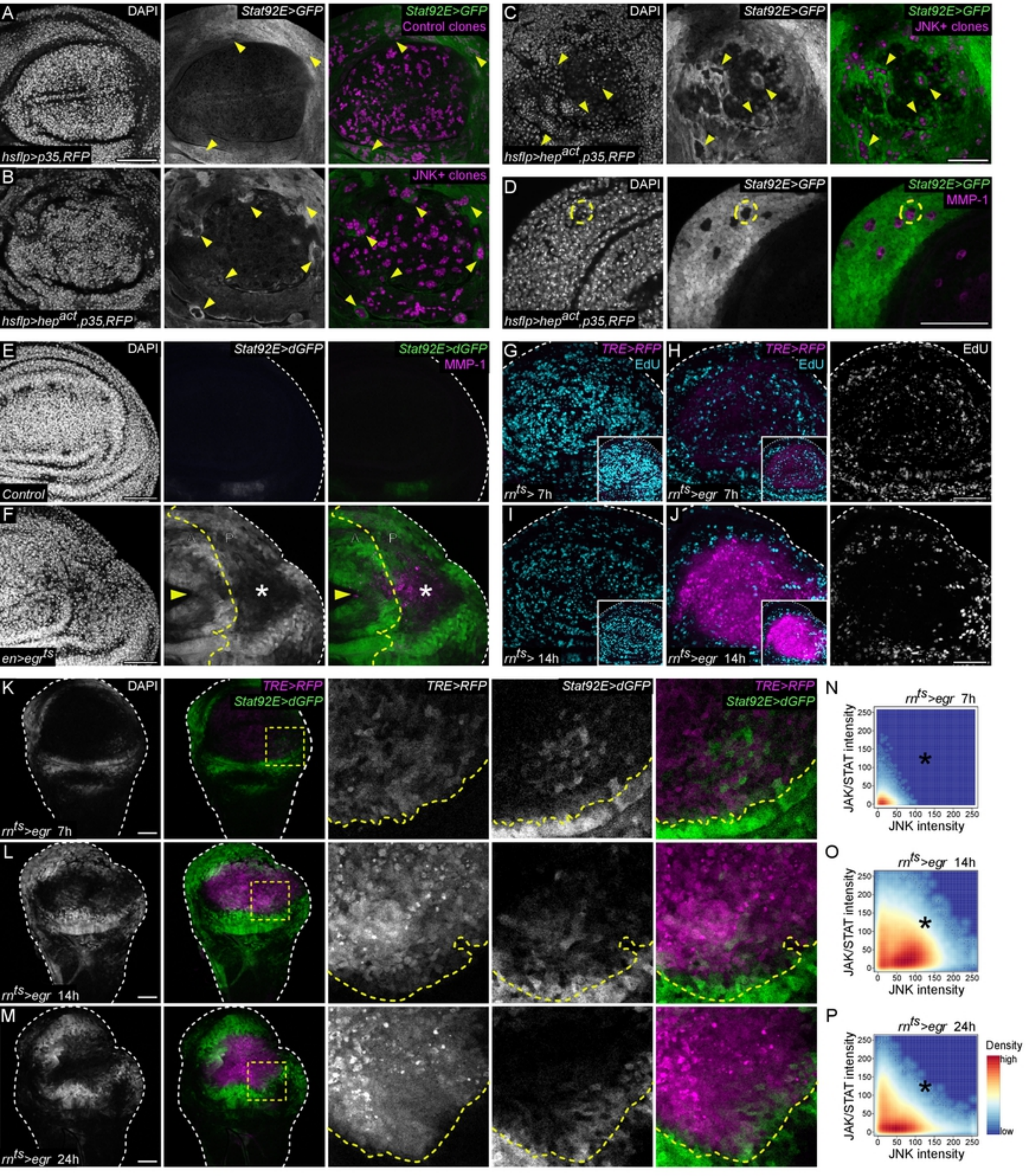

Figure 2

Figure 3

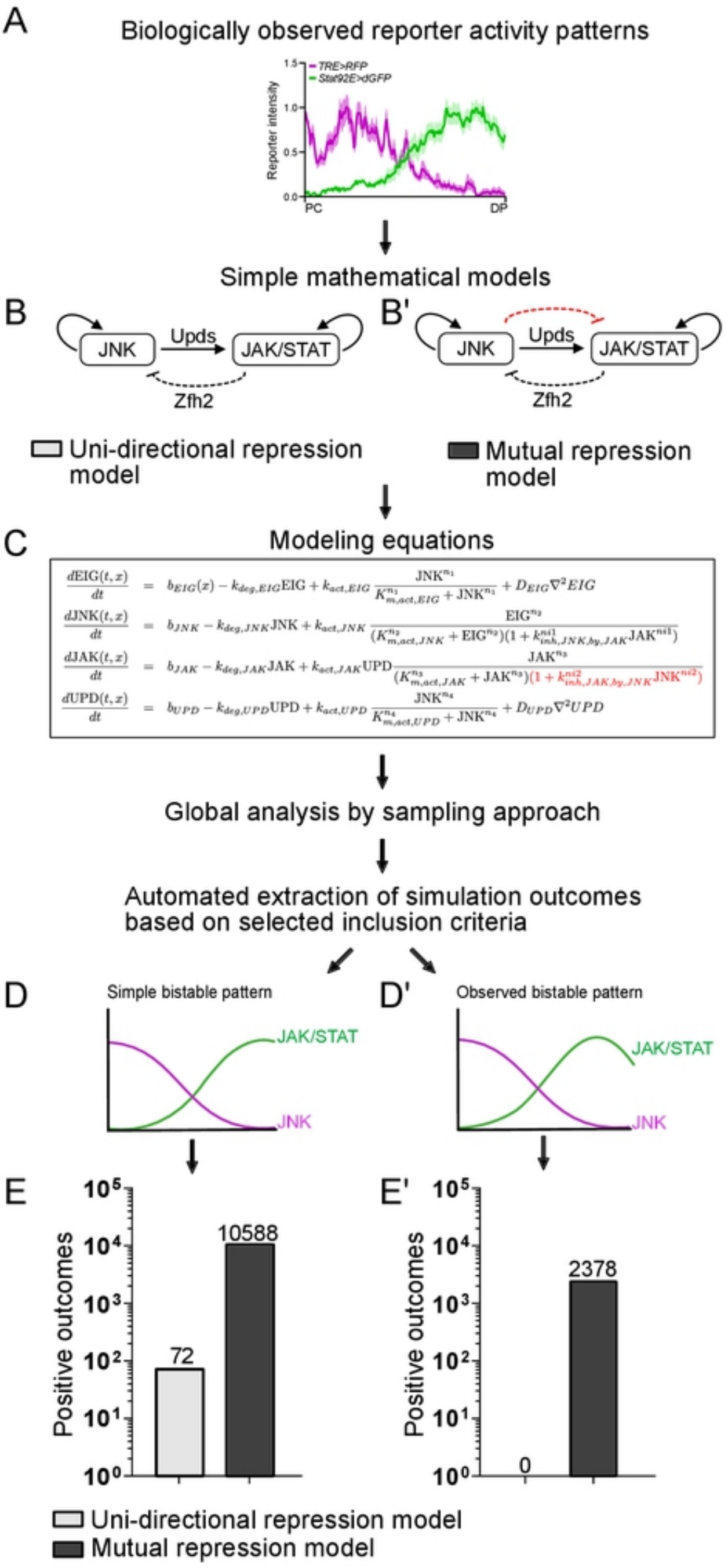

Figure 3

Figure 4

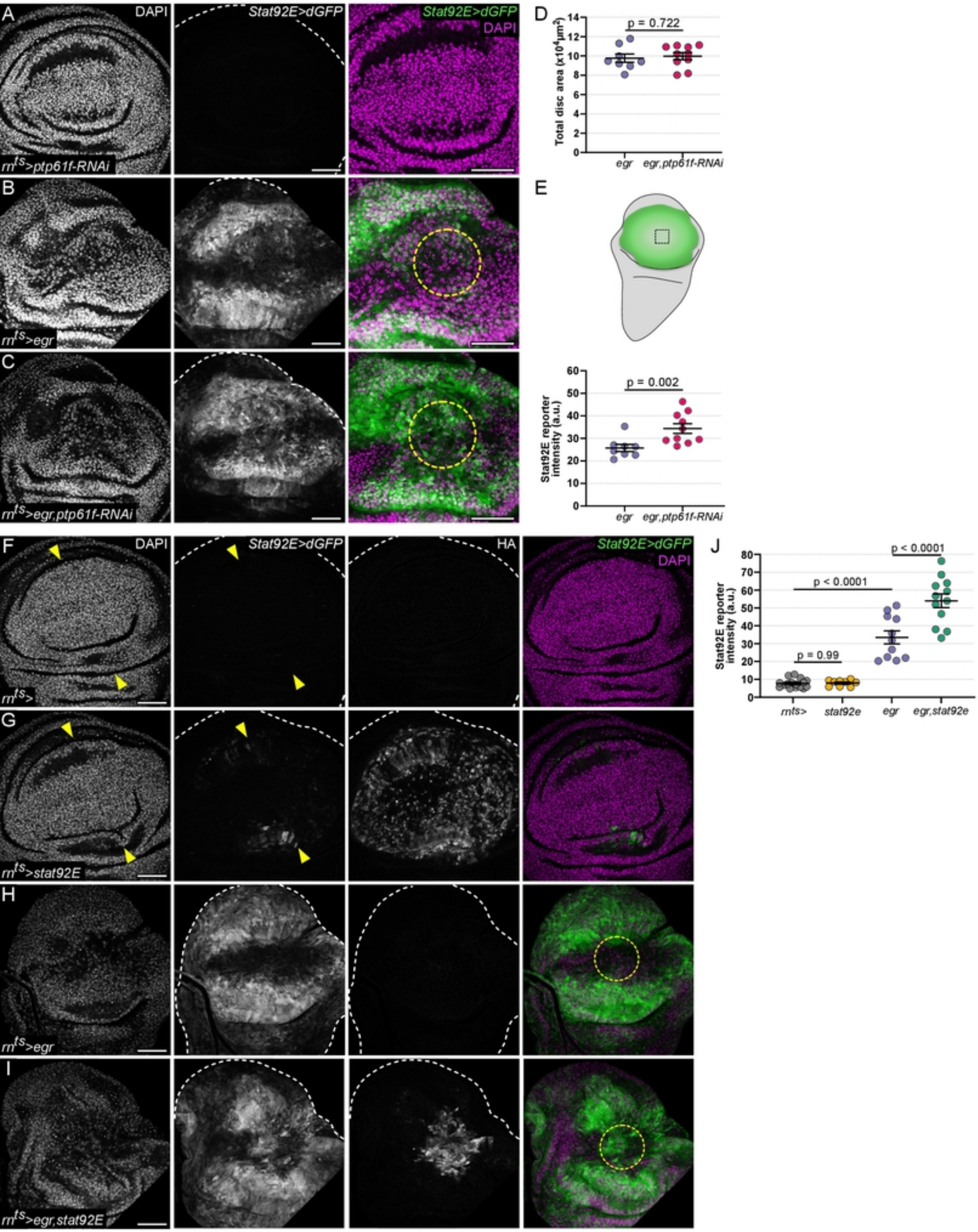

Figure 4

Figure 5

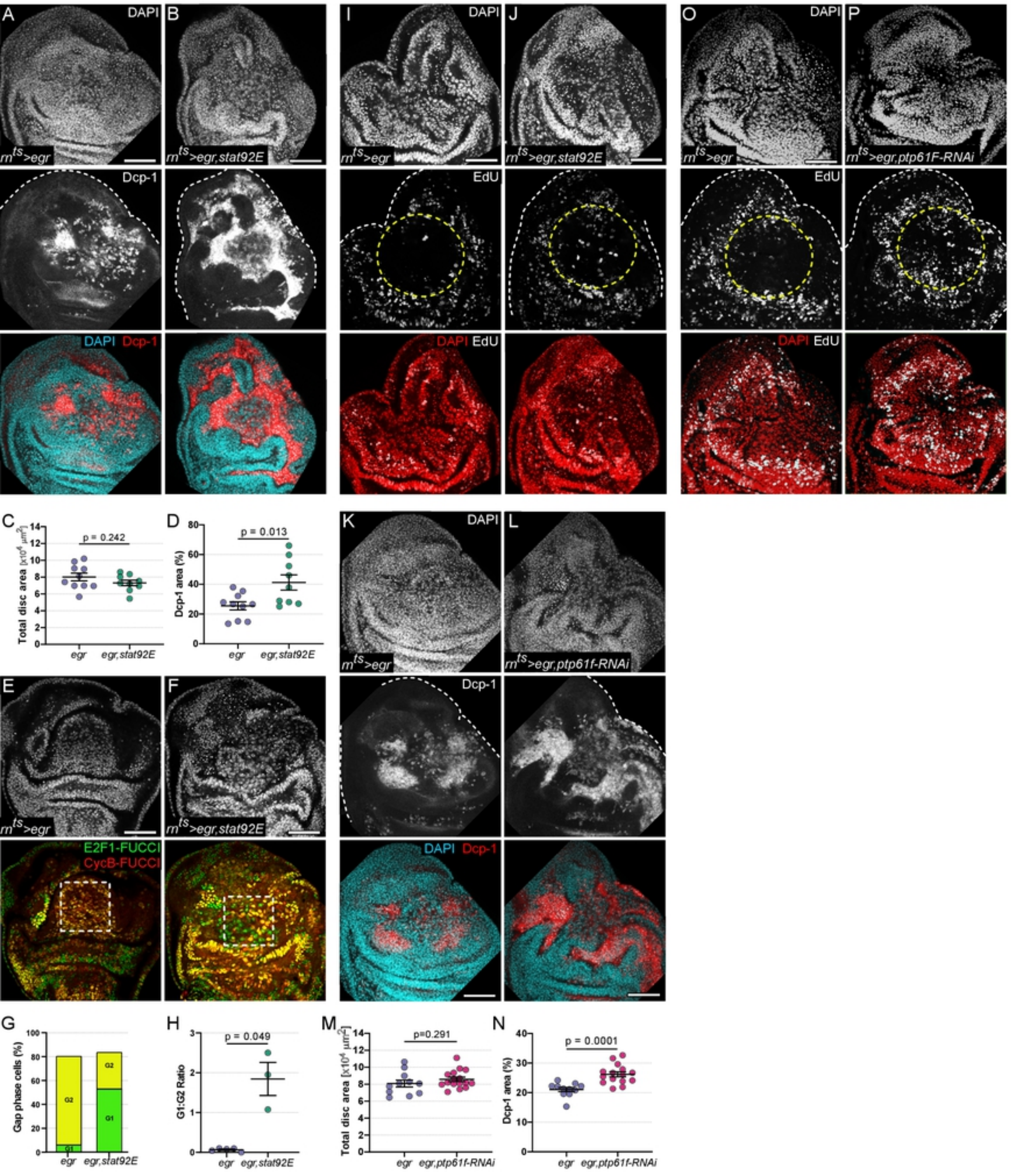

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Figure 6

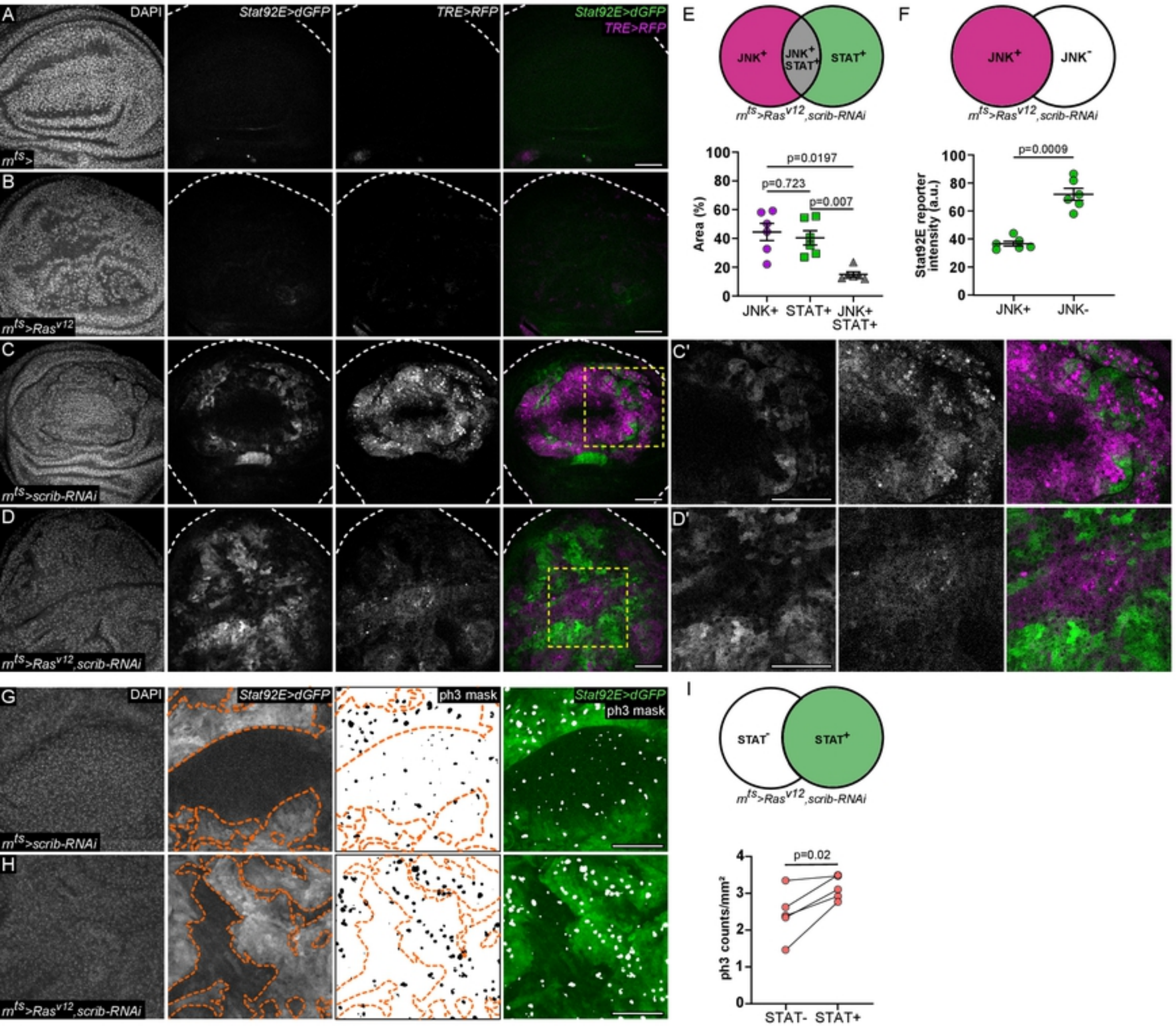

Figure 6

Figure 7

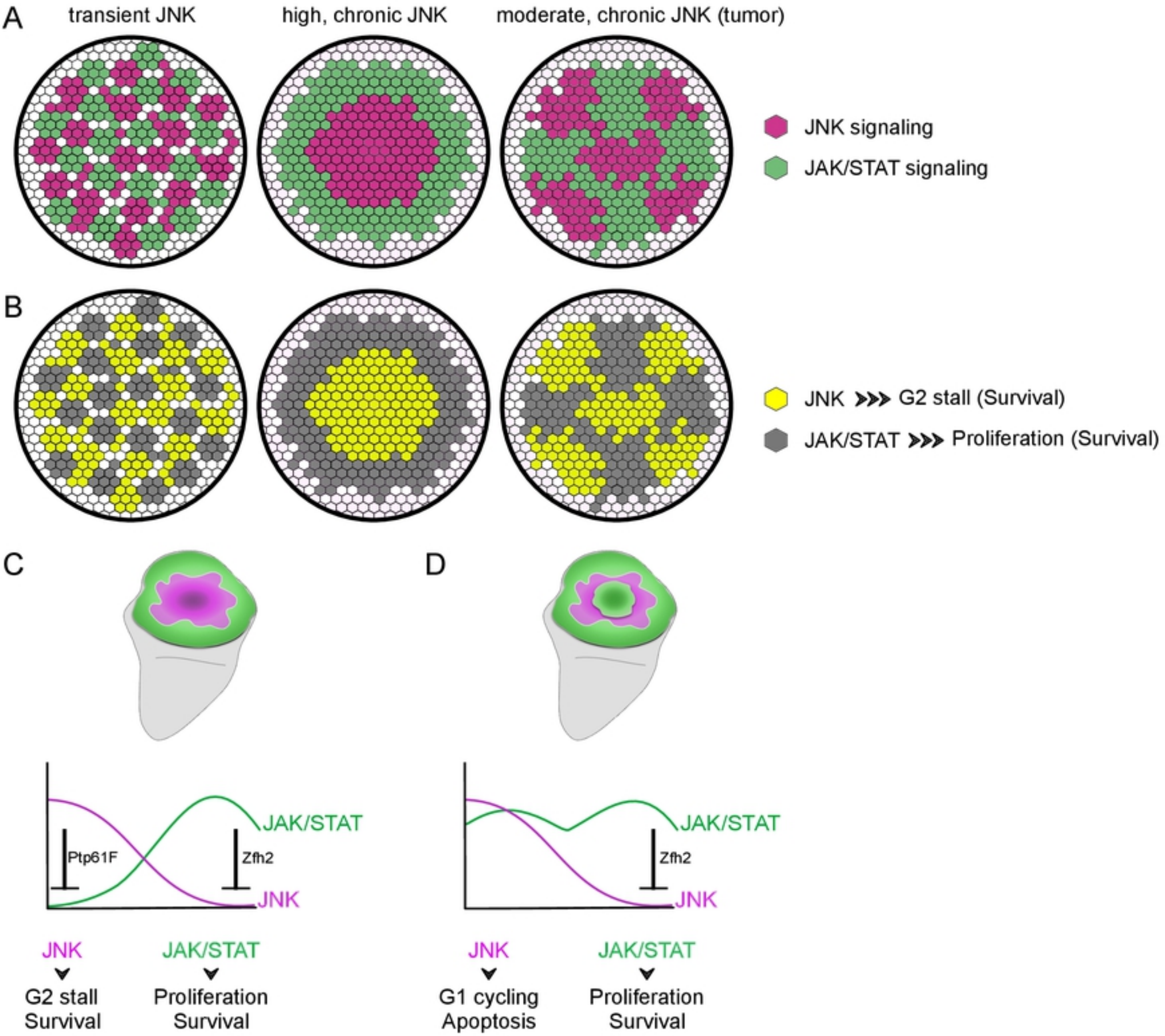

Figure 7