THE YKI-CACTUS ($I_K B\alpha$)-JNK AXIS PROMOTES TUMOR GROWTH AND PROGRESSION IN DROSOPHILA

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Running Title: Yorkie-Cactus-Jun Kinase axis in tumor growth

Abstract

Presence of inflammatory factors in the tumor microenvironment is well known yet their specific role in tumorigenesis is elusive. The core inflammatory pathways are conserved in *Drosophila*, including the Toll-Like Receptor (TLR) and the Tumor Necrosis Factor (TNF) pathway. We used *Drosophila* tumor models to study the role of inflammatory factors in tumorigenesis. Specifically, we co-activated oncogenic forms of Ras^{V12} or its major effector Yorkie (Yki^{3SA}) in polarity deficient cells mutant for tumor suppressor gene *scribble* (*scrib*) marked by GFP under *nubGAL4* or in somatic clones. This system recapitulates the clonal origins of cancer, and shows neoplastic growth, invasion and lethality. We investigated if TLR and TNF pathway affect growth of $Yki^{3SA}scrib^{RNAi}$ or $Ras^{V12}scrib^{RNAi}$ tumors through activation of tumor promoting Jun N-terminal Kinase (JNK) pathway and its target Matrix Metalloprotease1 (MMP1). We report, TLR component, Cactus (Cact) is highly upregulated in $Yki^{3SA}scrib^{RNAi}$ or $Ras^{V12}scrib^{RNAi}$ tumors. *Drosophila* Cactus (mammalian $I_KB\alpha$) acts as an inhibitor of NF_KB signaling that plays key roles in inflammatory and immune response. Here we show an alternative role for Cactus, and by extension cytokine mediated signaling, in tumorigenesis. Downregulating Cact affects both tumor progression and invasion. Interestingly, downregulating TNF receptors in tumor cells did not affect their invasiveness despite reducing JNK activity. Genetic analysis suggested that Cact and JNK are key regulators of tumor progression. Overall, we show that Yki plays a critical role in tumorigenesis by controlling Cact, which in turn, mediates tumor promoting JNK oncogenic signaling in tumor cells.

Key words: Cancer, Yki, Ras, Inflammation, Signaling pathway, Proliferation, Invasion

Introduction

Oncogenic forms of Ras are dominant drivers of tumor growth in a third of human cancers (1). Oncogenic Ras activates multiple downstream pathways like PI3K, RAF-MEK-MAPK, JNK, p38 MAPK during tumorigenesis (2). *Drosophila* tumor models of oncogenic Ras (Ras^{V12}) with *scribble* (*scrib*) – the apical basal polarity regulator (referred as $Ras^{V12}scrib^{-}$ tumors), show neoplastic growth, invasion and lethality (3, 4). In Ras^{V12} $scrib^{-}$ tumors, Eiger (Egr), the ligand of *Drosophila* TNF, induces Jun N-terminal Kinase (JNK) pathway to promote tumor cell proliferation (5, 6). In contrast, Egr mediates elimination of defective cells through JNK specifically in *scrib* mosaic clones (7). Inflammatory components from the Toll Like Receptor (TLR) and Tumor Necrosis Factor (TNF) pathways play a significant role in tumor progression through poorly understood mechanisms (8, 9). However, the complex signaling initiated by oncogenic Ras presents challenges to deciphering the role of key inflammatory signals in tumor growth and progression.

A key effector of oncogenic Ras signaling is the Hippo pathway transcriptional coactivator protein Yki (*Drosophila* YAP ortholog) (10-12). Furthermore, elevated YAP activity is linked to neoplastic behavior in cancer cells, and poor prognosis in several cancers (13-15). To study the role of these inflammatory pathways in tumorigenesis, we established a Yki dependent model by expressing activated form of Yki ($UASYki^{3SA}$) in cells where *scrib* is downregulated ($UASscrib^{RNAi}$) using the GAL4-UAS system or in 'flp-out' clones. $Yki^{3SA}scrib^{RNAi}$ tumors show key hallmarks of cancer such as sustained proliferation via JNK activation (pJNK), Matrix Metalloprotease 1 (MMP1) mediated invasion, and degradation of basement membrane due to loss of Laminin. Using these models, we show that the *Drosophila* $I_KB\alpha$ orthologue Cactus (Cact), a member of the TLR pathway (16), is upregulated in both $Yki^{3SA}scrib^{RNAi}$ and

71 *Ras^{V12}scrib^{RNAi}* clones. Downregulation of Cact (*UAS-cact^{RNAi}*) in the tumor clones results in
72 downregulation/suppression of pJNK and MMP1. We show that Cact acts upstream of JNK, and
73 JNK activation may occur independent of Wengen (Wgn) and Grindelwald (Grnd), the
74 *Drosophila* TNF Receptors that signal through the JNK pathway (17). Furthermore, we report a
75 novel Yki-Cact-JNK signaling axis that promotes tumorigenesis downstream of Yki activation
76 that is critical in promoting Ras or Yki mediated tumor growth. The Yki-Cact-JNK axis plays a
77 critical role, as downregulation of Yki or Sd (*Drosophila* TEAD family transcription factor), or
78 Cact or JNK disrupts this signaling axis and inhibits *Ras^{V12}scrib^{RNAi}* tumorigenesis.

79 **Results**

80 **Comparison of oncogenic Yki and Ras dependent tumor models**

81 We coexpressed oncogenic Yki (*UASYki^{3SA}*), *scrib^{RNAi}* (*UASscrib^{RNAi}*) and GFP
82 (*UASGFP*) using *nub-Gal4* in wing imaginal discs [*nub>GFPYki^{3SA}scrib^{RNAi}*] (**Fig. 1A**)(18).
83 Compared to wild-type [*nub>GFP*], the *Yki^{3SA}scrib^{RNAi}* larvae appeared bloated, with overgrown
84 wing discs (**Fig. 1A'**), entered an extended larval life, and were 100% pupal lethal. The dissected
85 wing discs revealed large neoplastic growths confined to the wing pouch (detected by GFP
86 expression) (**Fig. S1A**). These neoplasms were invasive (**Fig. 1B**), as tumor cells (GFP positive)
87 extruded to the basal side and breached the basement membrane labelled with Laminin (red)
88 (**Fig. 1B, B'**)(3, 19). The *nub>Yki^{3SA}scrib^{RNAi}* system produced massive multilayered tumors with
89 high lethality, therefore, in parallel we made heat-shock mediated 'Flp-out' clones driven by
90 *Act>y+>Gal4* (20, 21). The wild-type clones detected by co-expression of GFP
91 (*AyGal4>UASGFP*) in the epithelial monolayer showed jagged clone boundaries (**Fig. S1B,**
92 **C**)(22), whereas the *Yki^{3SA}scrib^{RNAi}* clones *(AyGal4>UASGFP,UASYki^{3SA},UASscrib^{RNAi})*
93 presented as large epithelial outgrowths with well-sorted smooth borders (**Fig. 1C**). Compared to

wild-type clones (thickness 11µm), the *Yki*^{3SA}*scrib*^{RNAi} clones are multilayered (23.17µm) (**Fig. S1C**). Under comparable experimental conditions, the *Ras*^{V12}*scrib*^{RNAi} larvae (120h AEL) looked thin and sluggish, and dissected imaginal discs were fragile, neoplastic, and comprised exclusively of GFP-positive cells. When the heat shock was reduced to 3min, *Ras*^{V12}*scrib*^{RNAi} 'flp-out' clones also showed multi-layered organization (**Fig. S1C**). JNK mediated pro-proliferative signaling promotes tumorigenesis (8, 23-25). Consistent with this, the JNK reporter *puc*^{E69}-lacZ (26) was induced in *Ras*^{V12}*scrib*^{RNAi} and *Yki*^{3SA}*scrib*^{RNAi} clones (**Fig. 1C, D**). Furthermore, pJNK levels are also upregulated in *Ras*^{V12}*scrib*^{RNAi} and *Yki*^{3SA}*scrib*^{RNAi} clones (**Fig. S1D, E**). Next, to assess invasive capacity we assessed MMP1expression in our tumor models (27-30). MMP1 expression is very low in wild-type clones (**Fig S1F**), however, MMP1 is robustly induced in both *Yki*^{3SA}*scrib*^{RNAi} and *Ras*^{V12}*scrib*^{RNAi} tumor clones (**Fig. 1E, F**). Quantification of the integrated pixel intensity of MMP1 expression in *Yki*^{3SA}*scrib*^{RNAi} clone showed a 4-fold increase compared to the surrounding normal cells (**Fig. 1G**). Taken together, these data suggest that *nub>Yki*^{3SA}*scrib*^{RNAi} and *Ay>Yki*^{3SA}*scrib*^{RNAi} clones recapitulate key tumorigenic features of the well-established *Ras*^{V12}*scrib*^{RNAi} model like increased proliferation, formation of multilayered invasive neoplasms capable of basement membrane degradation, and induction of JNK signaling. Using these models, we investigated the role of key inflammatory pathways (TLR and TNF) in tumorigenesis.

Drosophila **IκBα component, Cactus, is upregulated in the tumor clones**

Cact prevents TLR activation by associating with and blocking nuclear localization of Dorsal, the *Drosophila* NF-κB homologue (Dl) or Dorsal-related Immunity Factor (Dif), two effectors of the TLR pathway (16). First, we evaluated Cact expression in tumor cells (16). Cact is ubiquitously expressed, and Cact levels are not affected in wild-type and *scrib*^{RNAi} clones (**Fig.**

2A, B, quantified in **Fig. 2E**). However, *Yki^{3SA}* and *Yki^{3SA}scrib^{RNAi}* clones showed significant upregulation of Cact expression (**Fig. 2C, D** quantified in **Fig. 2E**). Likewise, both *Ras^{V12}scrib^{RNAi}* clones (**Fig. S2A,** quantified in **Fig. S2B**), and *nub>Yki^{3SA}scrib^{RNAi}* tumors in the wing discs (**Fig. S2C**) showed increased Cact expression. Taken together, our data suggests that tumor cells have increased Cact expression compared to surrounding normal cells. To test if Cact is required for tumorigenesis, we expressed *UAS-cact^{RNAi}* and checked if JNK activity is altered when Cact is downregulated in tumor cells. No significant changes in pJNK expression were seen in wild-type (*AyGal4>cact^{RNAi}*) (**Fig. 2F,** quantified in **Fig. S2D**) or in *scrib* mutant clones (*AyGal4>cact^{RNAi}scrib^{RNAi}*) (**Fig. 2G,** quantified in **Fig. S2D**). Interestingly, downregulation of Cact affected cell sorting but not clone size as the jagged clone boundaries in *scrib^{RNAi}* clones (**Fig. 2B)**, were altered to well-sorted smooth borders in *cact^{RNAi}scrib^{RNAi}* clones **(Fig. 2G)**. In *Yki^{3SA}cact^{RNAi}* (**Fig. 2H,** quantified in **Fig. S2D**) and *Yki^{3SA}scrib^{RNAi}cact^{RNAi}* clones (**Fig. 2I,** quantified in **J**), downregulation of *cact* prevented pJNK induction, and reduced pJNK expression to wild-type levels. Next, we evaluated the invasiveness of tumor clones when *cact* was downregulated. Downregulation of *cact* in wild-type (**Fig. 2K**) or *scrib^{RNAi}* (**Fig. 2L**, quantified in **Fig. S2F**) clones did not affect MMP1 expression. In *Yki^{3SA}cact^{RNAi}* clones MMP1 levels were significantly reduced (**Fig. 2M**) when compared to *Yki^{3SA}* clones, but were significantly higher than wild-type clones (**Fig. S2F, G**). Remarkably, MMP1 levels are reduced to wild-type in *Yki^{3SA} scrib^{RNAi}cact^{RNAi}* clones (**Fig. 2N,** quantified in **O**) although these clones may still be multi-layered (**Fig. S2E**). Overall, our data showed that decreasing levels of *cact* in the tumor cells reduced JNK activation and MMP1 induction, thereby affecting tumor growth and invasiveness. Interestingly, *nub>Yki^{3SA}scrib^{RNAi}cact^{RNAi}* wing discs also showed reduced pJNK (**Fig. S2H**) and MMP1 (**Fig. S2I**) expression when compared to *nub>Yki^{3SA}scrib^{RNAi}* wing

discs. The difference in pJNK and MMP1 reduction can be attributed to inherent differences in tumor induction mechanism *viz.*, by a short pulse of Flippase expression in the 'flp-out' clones versus sustained Gal4 driven expression. Overall, these data suggest that elevated levels of *cact* play a key role in tumorigenesis, and downregulation of *cact* affects tumor growth by downregulating JNK signaling.

TNF pathway affects tumor growth but not invasiveness

Upregulation of the *Drosophila* TNF ligand Egr is shown to induce JNK activity in $Ras^{V12}scrib^{RNAi}$ tumors (5, 6). Egr requires TNF receptors Wgn and /or Grnd for signaling, and downregulation of *wgn* can attenuate Egr activity (31, 32). Hence to manipulate Egr activity in tumor clones, we first downregulated *wgn* through RNA interference in the $Yki^{3SA}scrib^{RNAi}$. No significant change in pJNK(**Fig. 3A,** quantified in **E)** or MMP1 (**Fig. 3F,** quantified in **J)** expression was observed in clones expressing wgn^{RNAi}**,** or $scrib^{RNAi}wgn^{RNAi}$ (**Fig. 3B, G**). However, the size of $scrib^{RNAi}wgn^{RNAi}$ clones was significantly smaller than $scrib^{RNAi}$ clones (**Fig. S3A**). This is consistent with previous reports where *scrib,egr* double mutant clones grew poorly compared to *scrib* mutant clones (7). In $Yki^{3SA}wgn^{RNAi}$ clones pJNK upregulation is blocked (**Fig. 3C,** quantified in **Fig. S3B).** However, compared to Yki^{3SA} clones, MMP1 was robustly induced in $Yki^{3SA}wgn^{RNAi}$ clones (**Fig. 3H,** quantified in **Fig. S3C, D**). Similarly, in $Yki^{3SA}scrib^{RNAi}wgn^{RNAi}$ clones pJNK activation is suppressed (**Fig. 3D,** quantified in **E**). Interestingly, MMP1 is significantly upregulated in $Yki^{3SA}scrib^{RNAi}wgn^{RNAi}$ clones comparable to levels in $Yki^{3SA}scrib^{RNAi}$ clones (**Fig. 3I,** quantified in **Fig. 3J**). Similar findings were reported in $Ras^{V12}scrib^{RNAi}wgn^{RNAi}$ tumors or chromosomal instability induced tumor models where MMP1 induction is not affected by downregulation of *wgn* (17, 33). These data suggested that either the wild-type levels of

pJNK are sufficient for MMP1 induction in tumors, or Grnd, the other TNF receptor, regulates the activation of TNF-JNK pathway during tumor growth and invasion.

We could not generate *Act>y+>Gal4UASgrnd^{RNAi}* recombined lines since 'flp-out' clones made with *eyFLP* or *hsFLP* lacked a definitive phenotype. However, we succeeded in recombining *nubGal4 UASGFP* with *UASgrnd^{RNAi}* flies (details in supplementary methods). Similar to *wgn^{RNAi}*, pJNK levels were significantly reduced in wing discs from *Yki^{3SA}scrib^{RNAi}grnd^{RNAi}* larvae when compared to *Yki^{3SA}scrib^{RNAi}*, but significantly higher than wild-type (*nub>GFP*) levels (**Fig. S4A,** quantified in **Fig. 3K**). However, MMP1 was still significantly induced (**Fig. S4B** quantified in **Fig. 3L**). Previous reports show that tumors caused by chromosomal instability show high MMP1 induction despite *grnd* downregulation (33). To account for the possible functional redundancy between Wgn and Grnd, we downregulated both receptors and evaluated MMP1 expression in *Yki^{3SA}scrib^{RNAi}* tumors. Compared to *nub>Yki^{3SA}scrib^{RNAi}*, downregulation of both TNF receptors (*nub>Yki^{3SA}scribi^{RNAi}grnd^{RNAi}wgn^{RNAi}*) reduced pJNK levels significantly (**Fig. S4C,** quantified in **Fig. 3K)**, but no significant reduction in MMP1 expression was observed (**Fig. S4D,** quantified in **Fig. 3L**). Taken together, these data suggest that tumor growth but not invasiveness is regulated by TNF pathway receptors.

JNK regulates invasiveness but not Cact expression in the tumor

Although downregulating TNF receptors didn't affect MMP1 induction in the tumor cells (**Fig. 3K, L**), downregulating TLR component, Cact, reduced MMP1 expression to wild-type levels (**Fig. 2O**). Given that JNK is the key MAPK that can transcriptionally activate MMP1(27), we investigated if Cact can intracellularly activate JNK independent of the TNF receptors and cause MMP1 activation in tumor cells. We compared pJNK and MMP1 expression in the

185 *Yki*^{3SA}*scrib*^{RNAi} *wgn*^{RNAi} clones when JNK activity was inhibited by expressing dominant negative
186 form of *Drosophila* JNK, *bsk*^{DN} (34). As expected, downregulation of JNK in the *Yki*^{3SA} *scrib*^{RNAi}
187 clones (*Yki*^{3SA}*scrib*^{RNAi}*bsk*^{DN}) inhibited both pJNK activation (**Fig. 4A,** quantified in **D**) and
188 MMP1 induction (**Fig. 4E,** quantified in **G)**. Interestingly, co-expression of *bsk*^{DN} with
189 *Yki*^{3SA}*scrib*^{RNAi}*wgn*^{RNAi}, blocked both pJNK (**Fig. 4B,** quantified in **D**) and MMP1 induction (**Fig.**
190 **4F** quantified in **G**). Thus, in tumor cells MMP1 expression can be stimulated by a TNF
191 receptor-independent intracellular JNK activation mechanism. Furthermore, downregulation of
192 JNK and Wgn significantly reduced clone thickness (15.63µm) (**Fig. 4C**) as compared to that of
193 *Yki*^{3SA}*scrib*^{RNAi} tumors (23.17µm). Interestingly while Cact downregulation affected JNK
194 activation, downregulating JNK in tumor clones (*bsk*^{DN}*Yki*^{3SA}*scrib*^{RNAi}) showed significant Cact
195 expression similar to *Yki*^{3SA}*scrib*^{RNAi} tumor clones (**Fig. 4H,** quantified in **I**). Thus our data
196 showed, Cact acts upstream of JNK signaling, and affects oncogenic JNK signaling.

197 **Yki regulates Cact and JNK in promoting tumor survival and invasiveness**

198 Oncogenic cooperation can induce Yki and JNK activity in a context-dependent manner
199 (35, 36), and during anti-microbial response Yki can transcriptionally regulate Cact expression
200 (37). Given this, we investigated the role of Yki in regulating Cact and JNK levels in our tumor
201 models. We downregulated the levels of Yki (*UASyki*^{RNAi}) in *Ras*^{V12}*scrib*^{RNAi} tumor clones. Under
202 our experimental condition (5min heat shock at 37˚C to second instar larvae), clones expressing
203 *yki*^{RNAi} were competed out when the larvae were grown at 25˚C or 18˚C. Therefore, to prevent
204 elimination of *yki*^{RNAi} clones, we co-expressed p35- a pan caspase inhibitor for our experiments
205 (38). Co-expression of *p35* and *yki*^{RNAi} didn't affect Cact expression in the wild-type (**Fig. 5A**),
206 *scrib*^{RNAi} (**Fig. 5B**), or *Ras*^{V12} clones (**Fig. 5C**). In comparison to control clones (**Fig. 5A-C**), the
207 *Ras*^{V12}*scrib*^{RNAi}*yki*^{RNAi} clones survived, but showed significant reduction in both the clone size

(**Fig. 5E**) and thickness (**Fig. 5F**). Interestingly, in $Ras^{V12}scrib^{RNAi}yki^{RNAi}$ clones downregulation of Yki blocked Cact (**Fig. 5D,** quantified in **G**), pJNK (**Fig. S5A**) and MMP1(**Fig. S5B**) induction as compared to $Ras^{V12}scrib^{RNAi}$ clones.

The TEAD family transcription factor Scalloped (Sd) is the major binding partner for Yki mediated transcription (39-41). The Yki/Sd complex also regulates *cact* expression during innate immune response (37). Sd downregulation ($nub>sd^{RNAi}$) resulted in a diminished wing pouch and yielded adult flies with smaller wings (42). Cact levels are not affected in wing discs from $nub>sd^{RNAi}$ (**Fig. 5H**) or $nub>scrib^{RNAi}sd^{RNAi}$ larvae (**Fig. 5I**). High larval lethality was seen in $nub>Ras^{V12}sd^{RNAi}$ and $nub>Ras^{V12}scrib^{RNAi}sd^{RNAi}$ larvae grown at 25˚C. However, at 18˚C, Cact was not induced in $Ras^{V12}sd^{RNAi}$ (**Fig. 5J)** and $Ras^{V12}scrib^{RNAi}sd^{RNAi}$ wing discs (**Fig. 5K,** quantified in **L**). In addition, pJNK and MMP1 expression were also not induced in $Ras^{V12}scrib^{RNAi}sd^{RNAi}$ wing pouch (Fig. **S5C, D**). These results strongly support a model where Yki and Sd regulate Cact expression, and loss of either Yki or Sd is sufficient to prevent the upregulation of Cact in the tumor cells. Further downregulation of Yki, Sd or Cact is sufficient to cause downregulation of JNK-mediated sustained signaling that promotes proliferation and invasion. These results suggest a mechanism in which polarity deficient cells with activated Ras require Yki mediated Cact induction and JNK activation to drive tumor growth and invasiveness (**Fig. 6**).

Discussion

Yki dependent signals downstream of Oncogenic Ras promote tumor growth

Oncogenic Ras activation results in burst of signaling through downstream effectors (Raf, Rac, Rho, PI3K), which phosphorylate and activate transcription factors, such as c-Myc, c-Jun, and c-

Fos (27, 43). The plethora of downstream signals make it difficult to identify and analyze key signaling nodes that promote tumor growth. Recently, Hippo pathway effectors YAP/Yki have emerged as key effectors of activated Ras signaling (11, 12, 14, 15). Moreover, YAP/Yki are sites of signal convergence and integration in many mammalian cancers e.g., colon, pancreatic and lung cancer (13, 44). The JNK pathway has emerged as another tumor promoting mechanism that can exert both anti- and pro-tumor activities (24, 45). JNK signaling induces Yki activation during compensatory cell proliferation and neoplastic tumor growth (7, 36, 46, 47), and JNK suppresses Yki elevation in *scrib* mutant cells during growth regulation (48, 49). Thus, JNK, Yki, and their downstream transcription factors have emerged as synergistic drivers of tumor growth (35, 50-53).

Consistent with previous reports, overexpression of Yki^{3SA} alone leads to hyperplasia, and homozygous loss of *scrib* induces neoplasia (42, 54, 55). When $Yki^{3SA}scrib^{RNAi}$ are co-expressed, multilayered metastatic tumors formed that degrade the basement membrane (**Fig. 1)**. We confirmed that JNK signaling is activated in $Yki^{3SA}scrib^{-}$ induced neoplastic growth (**Fig. 1**). Polyploid giant cells in $Ras^{V12}scrib^{-/-}$ clones are linked to increased Yki and JNK activity that promotes tumor progression (56).Variation in cell size is usually seen in primary tumor cells whereas larger metastases show more uniform size (57). We found cells of variable size in the $Yki^{3SA}scrib^{RNAi}$ clones (**Fig.1C**) but uniform sized in *nub*>$Yki^{3SA}scrib^{RNAi}$ discs. Further, impaired Hippo signaling can induce JNK signaling via transcriptional activation of the Rho1-GTPase which mediates Yki-induced JNK activation and overgrowth (58). Similarly, in mammals, GPCRs can activate YAP/TAZ through RhoA suggesting Yki-Rho1-JNK axis involvement in bridging the two pathways (58, 59). Our data and other published work suggests that both Yki and JNK dependent signaling is activated in multiple tumor contexts including $Yki^{3SA}scrib^{RNAi}$

tumors. A key transcriptional target of JNK signaling is MMP1- a matrix metalloprotease required for tissue remodeling, wound healing, regeneration, and control of cell movement and cell adhesion during development; and degradation of the basement membrane during tumor invasion (25, 27-29, 60, 61). We used JNK activation and MMP1 induction as the criteria to evaluate tumor growth and invasiveness. The $Yki^{3SA}scrib^{RNAi}$ tumor clones showed high levels of JNK activity and MMP1 induction (**Fig. 1**) suggesting that the tumor cells have acquired invasive traits.

Yki regulates Cact and JNK in Tumor cells

Cact is a negative regulator of the *Drosophila* Rel/NFκB orthologues Dorsal, Dif and Relish. Degradation of Cact, allows NFκB activation and localization to nucleus where it regulates transcription of genes controlling inflammatory cytokines and cell growth (62, 63). However, we noticed an accumulation of Cact in our tumor clones. (**Fig. 2**). Downregulating Cact in tumor cells prevented tumor progression by suppressing JNK activation and MMP1 induction (**Fig. 2**). This indicated a role of Cact in promoting tumor progression. Increase in Toll signaling by downregulation of Cact aggravates JNK activated cell death (64). Interestingly, we observed a significant reduction in pJNK expression with downregulation of Cact in our tumor clones (**Fig. 2**). These observations indicate that IκBα can regulate proliferation through JNK. Moreover, we found that downregulating JNK by downregulating TNF receptors (**Fig. 3**) did not affect MMP1 induction in our tumor clone. Also, downregulation of JNK by expressing dominant negative form of JNK (**Fig. 4**), did not affect Cact expression in the tumor cells although it blocked JNK and MMP1 activation (**Fig. 4**). Based on these genetic interactions, we concluded that Cact acts upstream of JNK and plays a key role in regulation of oncogenic JNK signaling (**Fig. 6**). We also observed that Wgn downregulation in tumor clones did not affect

Cact accumulation (data not shown). Although JNK levels were reduced, MMP1 was still induced in such cells (**Fig. 3**). Since TNFR1 can antagonistically regulate cell cycle through JNK and NFκB (65), it will be interesting to explore whether such signaling crosstalk between these three pathways affects mammalian tumorigenesis.

JNK is known to play a context dependent role to promote tumor growth. Work from our lab and others found that, Yki can also regulate JNK activation in the tumor cells. Liu et al. demonstrated that Yki can regulate the TLR pathway and prevent antimicrobial response (37). They showed that in the *Drosophila* larval fat body, Yki along with its transcriptional co-activator Scalloped, is capable of activating Cact. We also observed that downregulating either Yki or Scalloped severely affected tumor growth and showed no overexpression of Cact (**Fig. 5**). Further, downregulating Yki in tumor cells also restored JNK level. Similarly, in Basal Cell Carcinoma YAP interacts with its DNA binding transcription factors, TEAD and promotes c-Jun activity while loss of YAP leads to reduction in phosphorylated JNK and JUN (66). Overall, based on these data we propose a new mechanism where Yki functions with Sd to activate Cact and regulate JNK induction in the tumor cells (**Fig. 6**). Although *cact* is a transcriptional target of the Yki/Sd complex during innate immune response (37), whether this interaction is involved in tumor immunogenicity remains unknown. Recent studies have shown that inactivation of Hippo pathway in tumor cells induces host inflammatory responses, and the pathway can respond to and mediate inflammatory signals (67, 68). In conclusion, our study unraveled the interaction between the Yki, JNK and Cact to drive tumorigenesis. Therefore, it will be interesting to explore the mechanisms underlying the role of innate immunity in tumor progression and metastasis.

Material and methods

Fly Strains and Generation of Clones: All fly mutant and transgenic lines are described in Flybase, and were obtained from the Bloomington Drosophila Stock Center unless otherwise specified. *UAS-GFP* labeled clones were produced in larval imaginal discs using the following strains: *nub-Gal4/Cy*O (from S. Cohen), *yw; Act>y+>Gal4,UAS-GFP* (BL4411), $UASYki^{3SA}$(BL28817), $UASscrib^{RNAi}$ (BL58085, BL59080), $UASwgn^{RNAi}$ (BL50594), $UASgrnd^{RNAi}$*/CyO* (from A. Bergmann), $UAScact^{RNAi}$ (BL31713), $UASRas^{V12}$ (BL5788), $UASbsk^{DN}$ (BL6409), $UASyki^{RNAi}$ (BL34067), $UASsd^{RNAi}$(BL29352),*UASP35*, and puc^{E69}*-lacZ*. Appropriate genetic crosses were performed to establish recombined fly stocks to generate clones of the indicated genotypes. To induce somatic clones, larvae (48 hr after egg laying) were heat shocked at 37˚C for 5 min. This heat shock regimen was followed to induce Yki^{3SA} $scrib^{RNAi}$ clones alone or in combination with other transgenes described in the manuscript.

Immunohistochemistry: Third instar larvae were dissected and processed for immunohistochemistry following standard protocol (69).The samples were mounted in the VectaShield mounting medium (Vector Labs) and scanned using confocal microscopy (Olympus Fluoview 1000, 3000). The primary antibodies used were mouse anti-Cact (DHSB, 1:200), rabbit anti-pJNK (Cell Signaling Technology, 1:250), mouse anti-β gal (DSHB, 1:250), rabbit anti-Laminin (Ab-Cam,1:250) and mouse anti-MMP1 (DHSB, 1:200). Secondary antibody used were donkey Cy3-conjugated anti-mouse IgG (1:200, Jackson ImmunoResearch), and donkey Cy3-conjugated anti-rabbit IgG (1:200, Jackson ImmunoResearch).

Quantification: The immunohistochemistry data was quantified using the measurement log function of Photoshop (Adobe Photoshop CC 2018). For the heat shock induced clones, 3 circular ROI of 50pixel radius were used per clone (GFP positive) and adjacent normal region (GFP-negative). For the *nubGAL4* driven experiments, 3 square ROIs of 100 pixel length were

used. The average value of the integrated density was compared in between the normal non-GFP and clone specific GFP positive cells. For comparison between two different genotypes, ratio of the average integrated density in the GFP positive and negative region was compared. A ratio of 1 indicated no change in expression level between the tumor (GFP positive) and normal (GFP negative) region. In all studies, the change in expression compared between different genotypes is normalized with wild-type levels set to 1. Thickness of the clones was measured using the inbuilt ruler of the Fluoview 3000 software in the XZ/YZ optical sections for all experiments. Statistical significance was quantified by Student t-test using Graphpad Prism 5 software.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary information is available at Oncogene's website.

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Figure legends

Figure 1: Expression of Oncogenic Yki (*Yki^{3SA}*) in *scrib* mutant cells forms invasive tumors

(**A-A'**) Mature 3rd instar larvae from *nub>GFP* (**A**) and *nub>Yki^{3SA}scrib^{RNAi}* (**A'**) show growth of wing discs (GFP, grey). (**B-B"**) Panels show (**B**) rabbit anti-Laminin (red) expression in *nub>Yki^{3SA}scrib^{RNAi}* (GFP, green) wing disc (60X magnification). The inset (**B'**) shows discs labelled by Laminin (red) to test motility of tumor cells (green). Panel (**B''**) shows the XZ and YZ sections of the panel (**B**) highlighting the invasive cell (arrow). (**C-F**) Expression of *puc^{E69}-lacz* tracked with mouse anti-β-gal antibody (red, grey) in (**C**) *Yki^{3SA}scrib^{RNAi}puc^{E69}* and (**D**) *Ras^{V12}scrib^{RNAi}puc^{E69}* clones (green). Panels show expression of MMP1 labelled with mouse anti-MMP1 antibody (red, grey) in (**E**) *Yki^{3SA}scrib^{RNAi}* and (**F**) *Ras^{V12}scrib^{RNAi}* clones (green). (**G**) Graph shows quantification of MMP1 expression in *Yki^{3SA}scrib^{RNAi}* tumor (GFP expressing clone) and normal cells (non-GFP expressing cells outside the clone). Paired student T test with n=5, 95% confidence was performed using Graphpad Prism 5, $p < 0.0001$.

Figure 1: Role of Cact in tumor progression

Panels show Cact expression (red, grey) in GFP-labelled clones (Green) from (**A**) *AyGal4>GFP*, (**B**) *AyGal4>scrib^{RNAi}*, (**C**) *AyGAL4>Yki^{3SA}* and, (**D**) *AyGAL4> Yki^{3SA}scrib^{RNAi}* wing discs. (**F-I**) pJNK (red, grey) and (**K-N**) MMP1 expression (red, grey) in clones (green) when Cact is downregulated. (**E, J, O**) Bar graphs depict quantification of change in Cact (**E**), pJNK (**J**) and MMP1 (**O**) expression between indicated genotypes. *= $p<0.05$, ns= not significant

Figure 2: Effect of downregulating both the TNF receptors on the Tumor progression

Panels show wing imaginal discs containing heat-shock induced GFP labelled (green) clones expressing *wgn*^{RNAi} in the indicated genotypes stained with Rb anti-pJNK antibody (**A-D**) or anti-MMP1 antibody (**G-J**). (**E, J-L**) Bar graphs showing quantification of pJNK (**E, K**) and MMP1 (**F, L**) expression amongst indicated genotypes. *= p<0.05, ns= not significant

Figure 4: JNK is the key regulator of tumor invasiveness

Panels (**A-B', E-F', H-H'**) show GFP-labelled heat-shock clones (green) in the wing imaginal discs of *Yki*^{3SA}*scrib*^{RNAi}*bsk*^{DN} (**A, E, H**) and *Yki*^{3SA}*scrib*^{RNAi}*bsk*^{DN}*wgn*^{RNAi} (**B, F**) stained for antibodies against pJNK (red, grey) (**A, B**), MMP1 (red, grey) (**E, F**), and Cact (red, grey) (**H**). (**C**) Thickness of the clones representing the multi-layered structure of indicated genotypes was measured using Photoshop, n=8. (**D, G, I**) Bar graphs show quantification of change in expression of pJNK (**D**), MMP1 (**G**) and Cact (**I**) amongst indicated genotype. *= p<0.05, ns= not significant

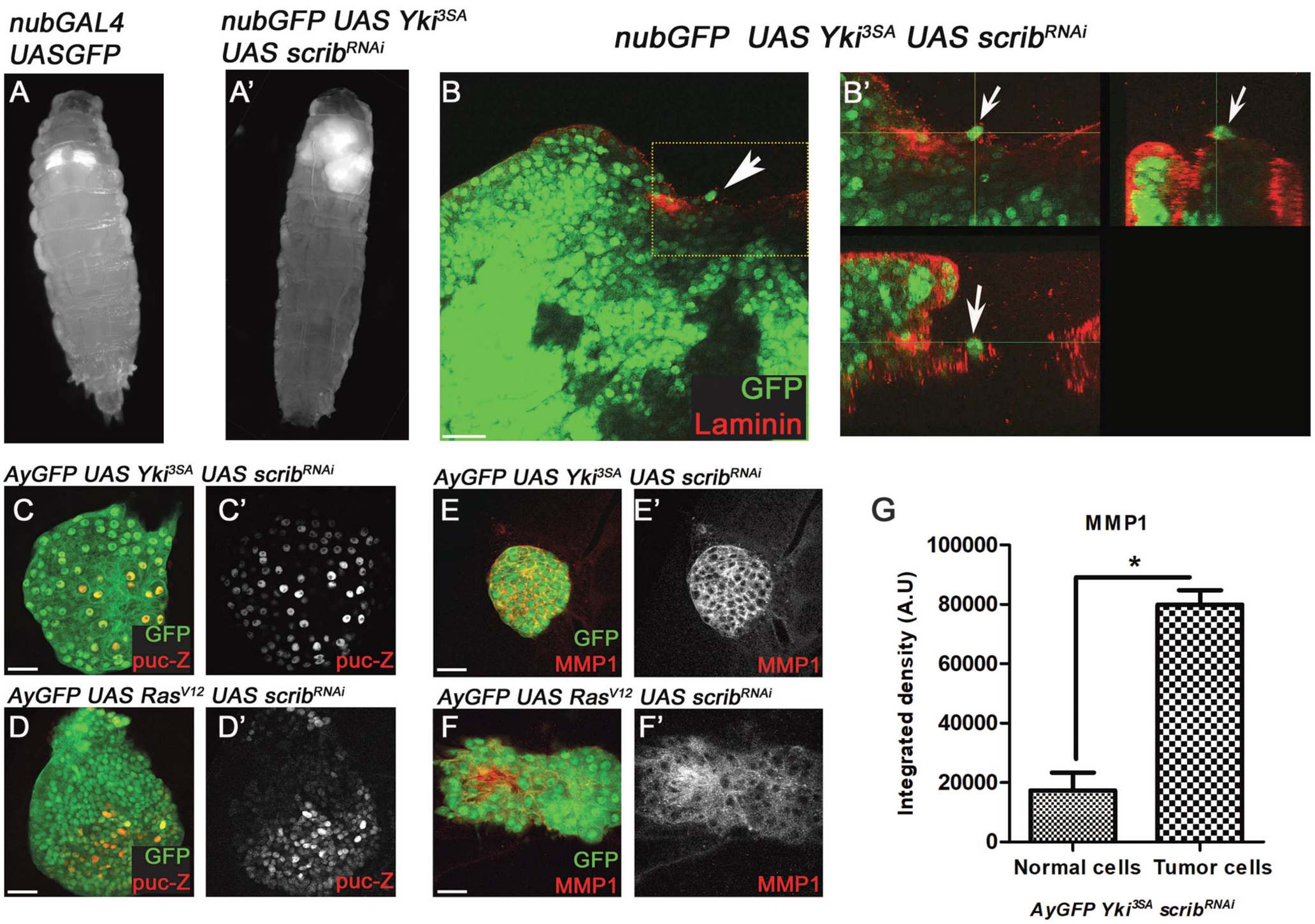
Figure 5: Role of Yki in stimulating Cact and promoting tumorigenesis

Panels **A-F** show effect of downregulating *yki* (*UASyki*^{RNAi}) in the GFP-labelled heat-shock clones (green) in wing imaginal discs from larvae of the indicated genotype. (**A-D**) Confocal images present effect of *yki*^{RNAi} on Cact (red, grey) expression. (**E-G**) Graphs show quantification of clone size (**E**), clone thickness (**F**), and change in Cact expression levels (**G**). Panels **H-L** show effects of *sd* downregulation (*UASsd*^{RNAi}) on *nubGal4* driven tumor phenotypes in the wing imaginal discs. (**H-K**) Confocal images of wing discs from indicated genotypes stained with anti-Cact antibody (red, grey) are presented. (**L**) Graph showing quantification of changes in Cact expression. *= p<0.05, ns= not significant

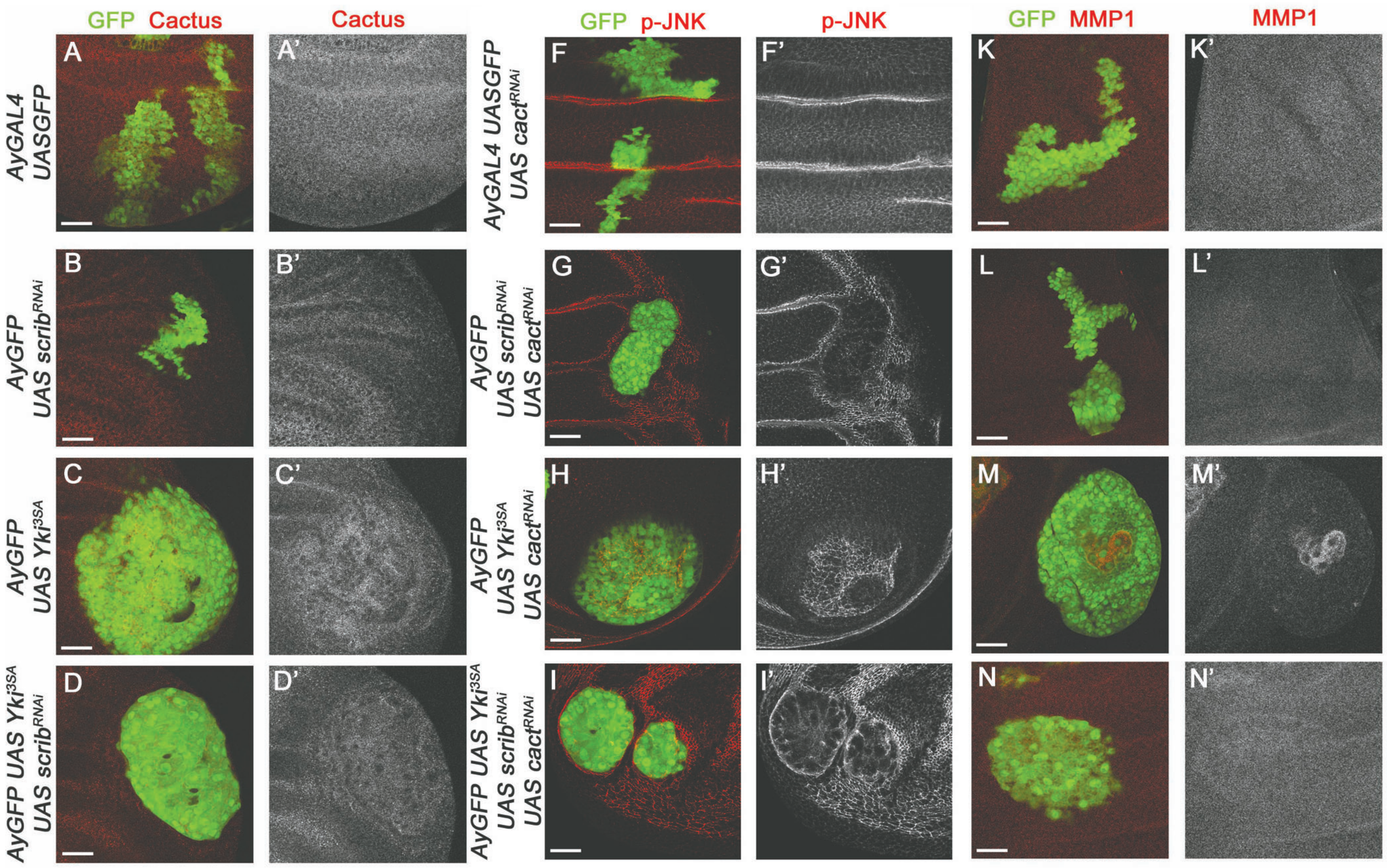
552

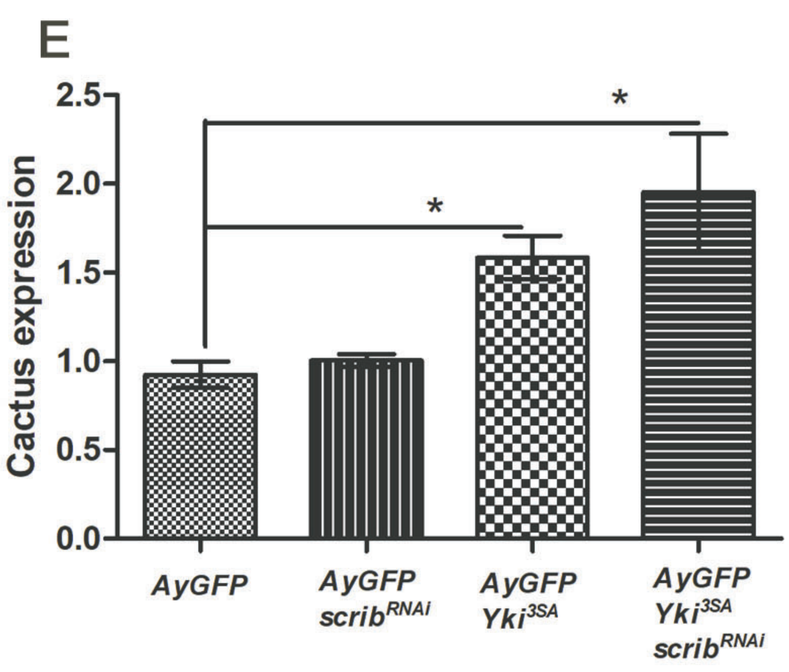
553 **Figure 6: Model for Yki mediated Cact and JNK activation promoting tumorigenesis**

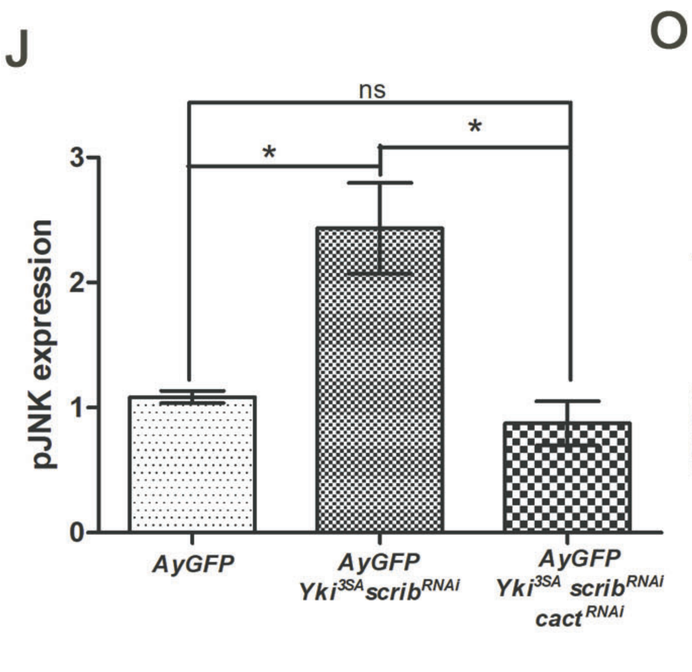
554 A model depicting the downstream effects of the cooperation between activated oncogene (*Yki* or
555 *Ras*) and loss of polarity gene, *scrib* is shown. In somatic clones (elliptical cells), JNK is
556 activated by TNF pathway ligand Egr and its receptors, Wgn and Grnd. Independently, JNK can
557 be activated in the tumor cells due to Cact accumulation via Yki activation. Yki mediated Cact
558 and JNK activation is critical for tumor progression.

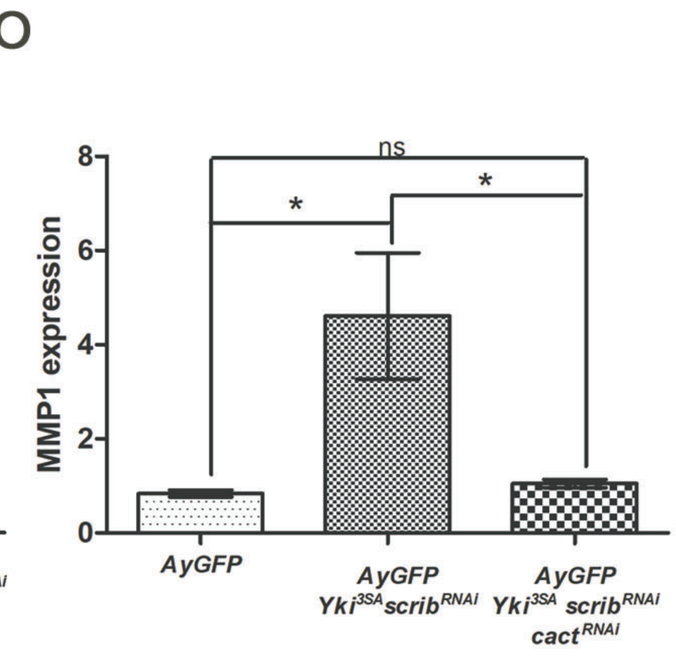


Snigdha et al Figure 1

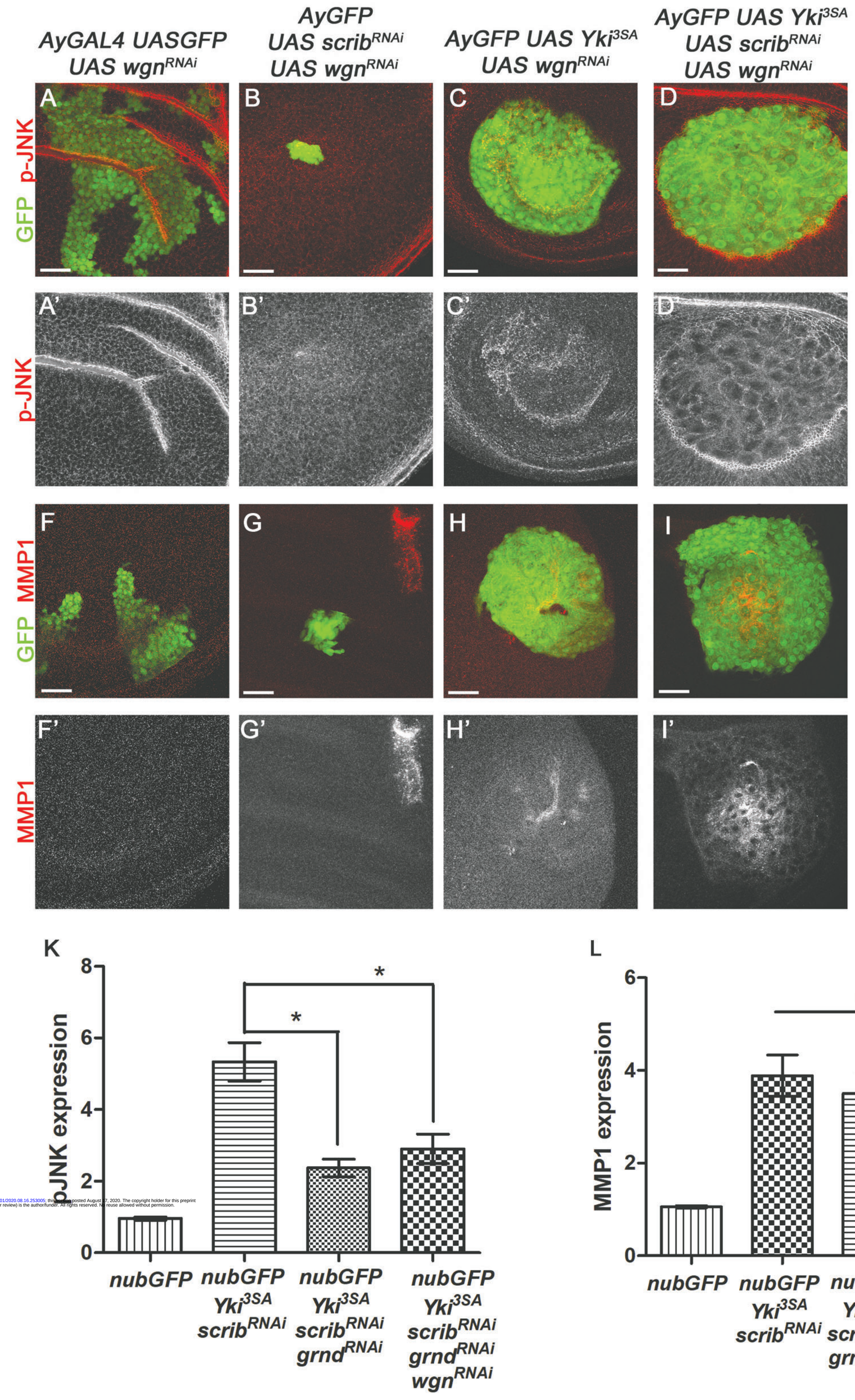




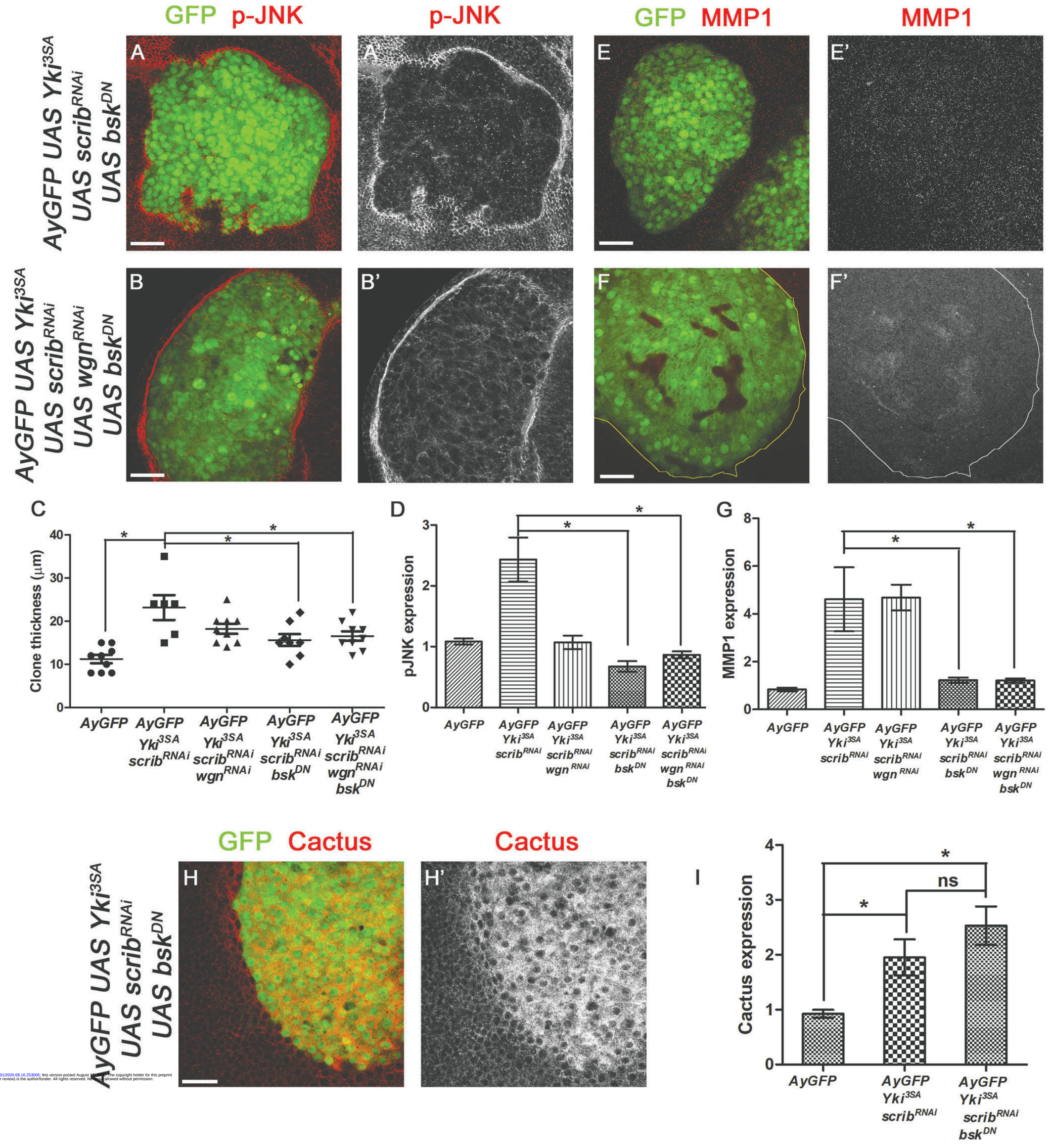




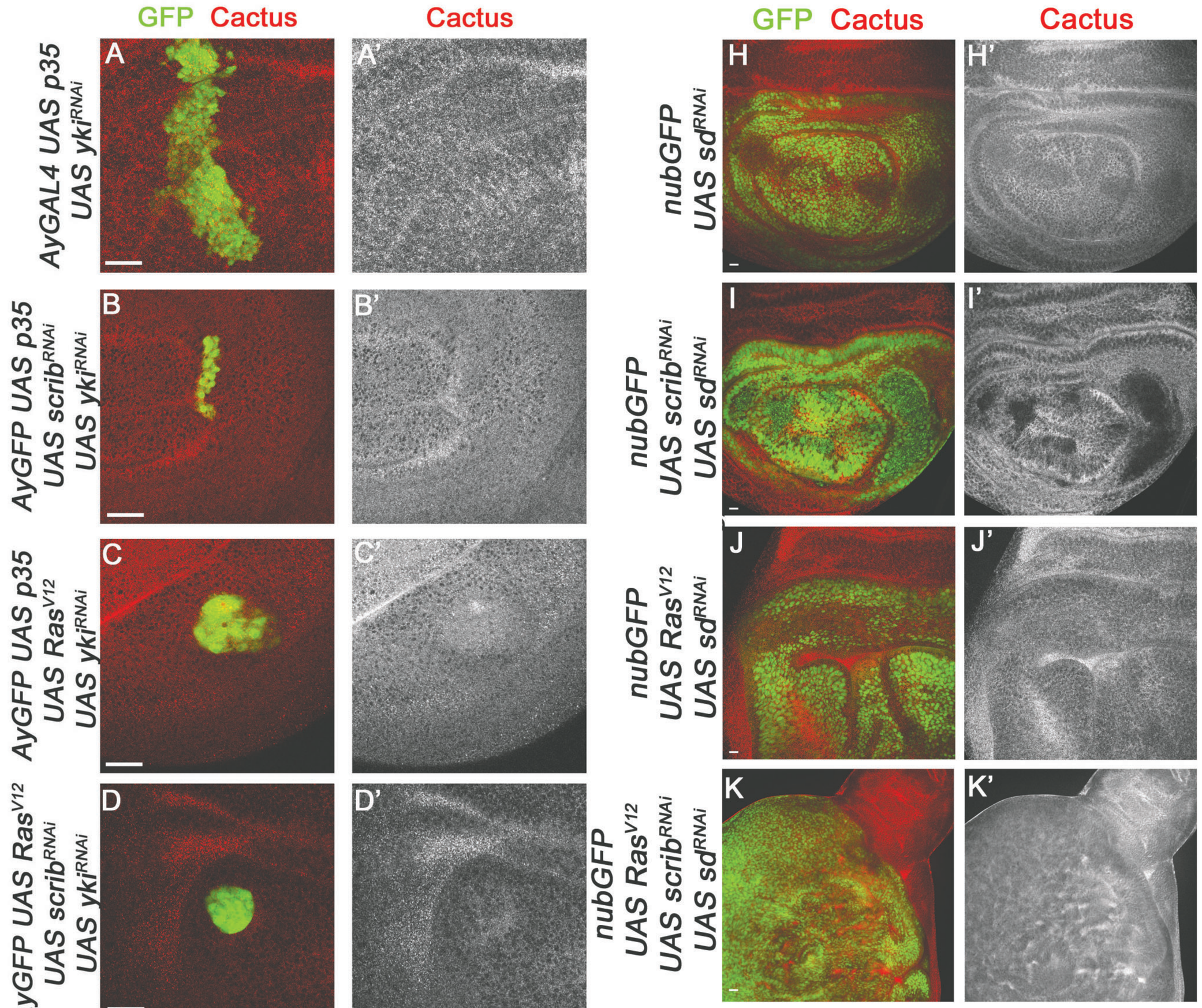
Snigdha et al Figure 2

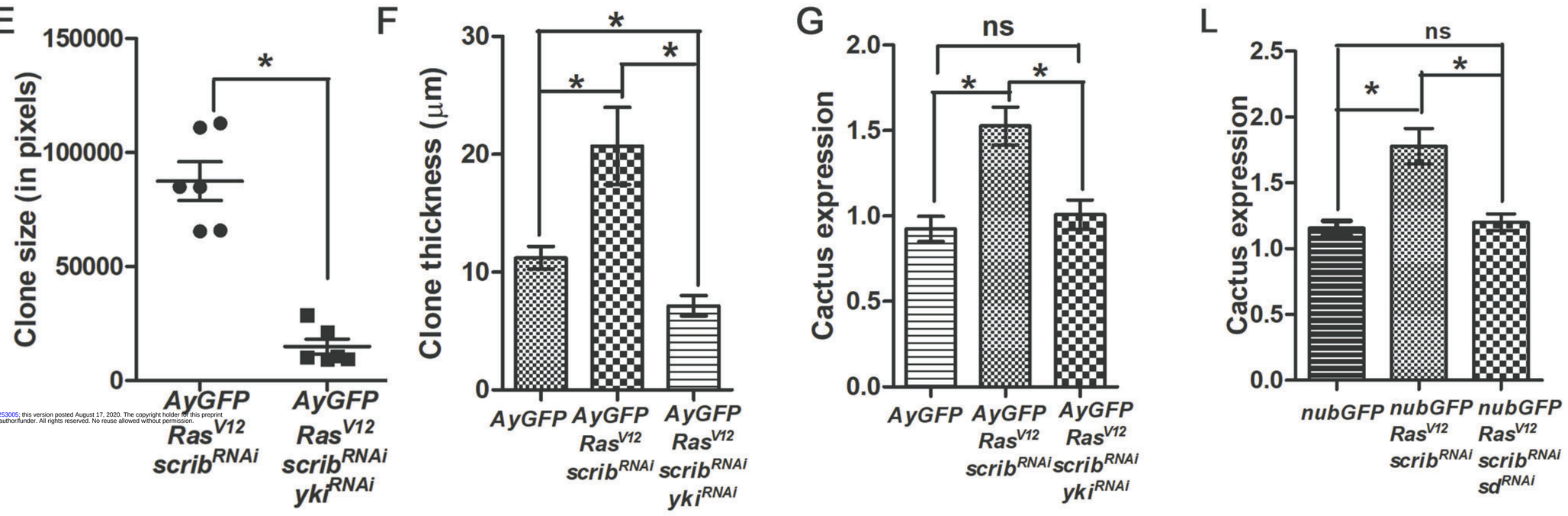


Snigha et al Figure 3

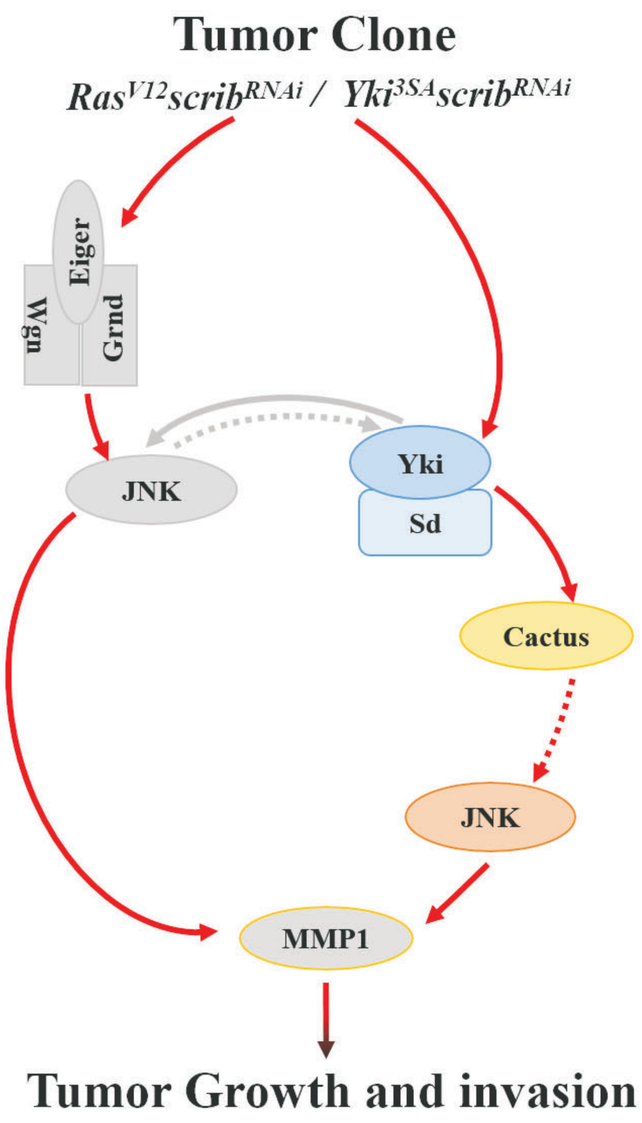


Snigdha et al., Figure 4





Snigdha et al Figure 5



Snigdha et al Figure 6