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A *Drosophila* model to study the functions of TWIST orthologs in apoptosis and proliferation

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Abstract

The *twist* gene has been characterized for its role in myogenesis in several species. In addition, in mammalian cultured cells, it has been shown that *twist* is a potential oncogene antagonizing p53-dependent apoptosis. To study, *in vivo*, the role of *twist* in apoptosis and proliferation, we constructed transgenic *Drosophila* lines allowing ectopic expression of different *twist* orthologs. We report that: (i) *Drosophila twist* induces apoptosis and activates the *reaper* promoter, (ii) nematode *twist* induces arrest of proliferation without apoptosis, and (iii) human *twist* retains its potentialities observed in mammalian cultured cells and antagonizes *Drosophila* p53-dependent apoptosis. In addition, we show that human *twist* is able to induce cell proliferation in *Drosophila*. Data suggest that the pathway by which human *twist* antagonizes *Drosophila* p53 could be conserved. These transgenic lines thus constitute a powerful tool to identify targets and modifiers of human *twist*.

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Abbreviations: bHLH, basic helix–loop–helix; MTWIST, mouse TWIST; IGF-1, insulin-like growth factor 1.

Introduction

The formation of a pluricellular organism needs the collaboration between two cellular processes: cell proliferation and cell differentiation. The basic helix–loop–helix (bHLH) proteins are transcription factors acting in various differentiation processes, either as positive or negative regulators, and play key roles in different developmental events like neurogenesis and myogenesis (for a review see Yun and Wold[1]). In general, they have been well conserved during evolution.

The *twist* gene encodes a bHLH factor that regulates distinct cell fate decisions in mesodermic tissues, especially during myogenesis, in species as distinct as *Drosophila*, nematode and human. The TWIST protein is conserved between *Drosophila*, *Caenorhabditis elegans* (*C. elegans*), and human, mainly at the level of the bHLH motif that contains the DNA-binding and protein–protein interaction domains.[2,3] A WR motif can also be identified in human TWIST, but is less conserved in flies, and not recognizable in nematodes.[4] The other domains are significantly divergent, which could explain the divergence observed in TWIST function in these different species (see Figure 1 and Castanon and Baylies[4]).

In *Drosophila*, the role of *twist* has been extensively studied.[3,5,6] It was first identified based on its function during gastrulation. Later, it plays a key role in executing subdivision of the mesoderm, and regulates determination of the somatic muscle lineage.[7] In late embryonic development, *twist* expression declines persisting only in myoblasts that will form adult muscles in later stages of development.[8] These cells migrate on imaginal discs and proliferate. In pupae, *twist*-expressing myoblasts are involved in adult muscle formation.[9] Interestingly, *twist* expression then fades suggesting the possibility that *Drosophila* TWIST is required to maintain the myoblasts in a proliferative state.

In *C. elegans* only one *twist* homolog, *hlh8*, which encodes the CeTWIST protein, has been identified and it is expressed in mesodermal cells forming the embryonic-born enteric muscles and the postembryonic M mesoblast lineage.[10] The characterization of a null mutant for *hlh8* allowed the identification of a requirement for *hlh8* in the cells where it is expressed, and, in addition, a later role in formation of nonstriated sex muscles.[11] Unlike *Drosophila twist* null mutants, null *hlh8* mutants are viable. Data indicate that in contrast to *Drosophila* TWIST, CeTWIST is not required for retaining myoblasts in a proliferative state.[11]

In Vertebrates, *twist* is known for its capacity to interfere with other bHLH proteins that control skeletal muscle development: MyoD, Myf5, myogenin, and MRF4. It has been shown that mouse *twist* (Mtwist) is able to inhibit skeletal myogenesis by blocking the activity of the myogenic bHLH and MEF2 family members.[12,13] This and other results indicate that, in Vertebrates, *twist* is clearly an inhibitor of myogenesis. Null Mtwist mice die at about 11 days showing cranial neural tube defects, disorganization of the somites, and a high degree of apoptosis.[14] In human, a role for *twist* in osteogenesis has been implicated as different mutations in the bHLH domain of *twist* are associated with the Saethre–Chotzen syndrome.[15–18]

Thus, the role of *twist* in myogenesis in different species seems to be well established but clearly with differences: activator role in *Drosophila* and on the contrary inhibitor role in Vertebrates. Nevertheless, a common feature between *Drosophila* and humans seems to be the expression of *twist* gene in proliferating tissues.

Recently, a new role for *twist* has been reported. Maestro *et al*.[19] showed that *twist* could be considered as a potential oncogene that inhibits apoptosis induced by c-myc or activated p53. In rat cells, Myc-induced apoptosis is reduced by 50% by *twist* and *twist* can prevent cell death through a

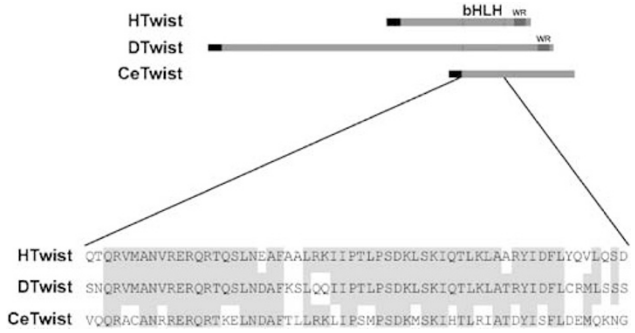


Figure 1 The different TWIST proteins. The human, nematode, and drosophila proteins differ in length, and in sequence except for the level of the bHLH domain. The NH2-terminal part of the protein is also conserved (in black), and *Drosophila* and human share a WR motif at the COOH terminus

variety of mechanisms.[19] The authors suggest that the antiapoptotic properties of TWIST may result from antagonism of the p53 pathway. Indeed, Vertebrate TWIST can counteract the transcriptional activation of p53 targets like p21, MDM2, and Bax. This effect is mediated by a significant downregulation of expression of the ARF tumor suppressor gene that antagonizes the MDM2 oncogene which, in turn, is a negative regulator of p53.[19]

Furthermore, it has been shown that the insulin-like growth factor I (IGF-1) induces *twist* expression in NIH-3T3 cells, and this is involved in the antiapoptotic effects of the IGF-1 receptor, suggesting a possible link between the IGF-1 and p53 pathways through *twist*.[20]

These data indicate that Vertebrate TWIST is involved in processes like proliferation and p53-dependent apoptosis. This raises several questions: (i) Are these potentialities conserved by the TWIST protein of other species? (ii) Do these functions of Vertebrate TWIST exist *in vivo* or are they only linked to the experimental procedure used (cultured cell lines)? (iii) Is the antiapoptotic function of Vertebrate TWIST restricted to a specific cell type or is it an intrinsic capacity of the vertebrate TWIST product?

In order to address these questions, we evaluated the effects of three different TWIST proteins (drosophila, nematode, and human) in induction of apoptosis and cell proliferation. We decided to test the role of the different *twist* genes in the same organism, *Drosophila melanogaster*, inducing expression of the three transgenes ectopically by the UAS/GAL4 system. This method has provided a powerful genetic system to elucidate cellular pathways. More particularly, it has been successfully applied to study human degenerative diseases (for review see Chan and Bonini[21]). Thus, we expressed ectopically the different TWIST proteins in a highly proliferative tissue, the wing imaginal disc, where *twist* is not normally expressed. This allowed us to avoid the possibility of the ectopic TWIST acting as a dominant negative with respect to the endogenous TWIST protein.

Here, we report that the ectopic expression of the three different *twist* genes using transgenic *Drosophila* lines induces a variety of responses that are very different for the three TWIST orthologs tested. This validates the use of transgenic animals expressing heterologous products to study gene function. The *Drosophila twist* induces apoptosis. The nematode *twist* induces a decrease in proliferation without apoptosis. The human *twist* induces both inhibition of p53-dependent apoptosis and cell proliferation. The results indicate conservation of the function of human TWIST when expressed in *Drosophila* and suggest possible new roles for the *twist* products of the other species. Moreover, they show that the p53 pathway in *Drosophila* responds to human *twist*, even if the other components of the pathway in mammals, like MDM2 and ARF, are either absent or not yet identified in *Drosophila*.

Results

Phenotypes induced by ectopic expression of *twist*

To test a possible role for *twist* from different species in apoptosis and proliferation, we used the UAS/GAL4 system of *Drosophila*. *C. elegans* and human transgenic lines were obtained, and will be called *UAS-twistCE* and *UAS-twistH*, respectively (see Materials and Methods). We chose to work with transgenic lines that are homozygous viable like the drosophila transgene, *UAS-twistD* (a gift of M Baylies).

We first tested by RT-PCR whether these transgenes are transcribed in wing imaginal discs when expressed under the control of the *MS1096-GAL4* driver (see Materials and Methods and Figure 2a). These tests proved positive for all three transgenes (data not shown). For the *UAS-twistH* transgene, using human TWIST antibody, we determined that human TWIST presents a nuclear localization (data not shown).

We next overexpressed the different transgenes using two strong drivers, *daugtherless-GAL4 (da-GAL4*) and *patched-GAL4* (*ptc-GAL4*), to evaluate their strength. *UAS-twistD* and *UAS-twistCE* gave 100% mortality with both drivers, while the *UAS-twistH* increased by 25% the mortality of the control (cross by Canton strain).

Finally, we ectopically expressed the different transgenes in eye and wing imaginal discs, where endogenous *twist* is not expressed, using *eyeless-GAL4* (*eye-GAL4*) and *GMR-GAL4* drivers for the eye and *MS1096-GAL4* and *vestigial-GAL4* (*vg-GAL4*) for the wing (Figure 1a and see Materials and Methods for the description of the drivers).

Similar strong wing phenotypes were observed for the *Drosophila* and *C. elegans* transgenes characterized by small size and poor differentiation (Figure 2b–d), while no effect was observed with the human transgene. This indicates that *twistCE* and *twistD* transgenes perturb normal proliferation and/or differentiation in the wing imaginal disc. Ectopic expression of either the *Drosophila* (Figure 2e,f) or nematode (Figure 2e,g) transgenes using the *eye-GAL4* driver caused an almost complete disappearance of the eye structure. Again no effect was observed with the human transgene.

The effects of *twistD* and *twistCE* on proliferating cells of the discs could be because of either: (i) an effect of ectopic TWIST on its own, or (ii) the formation of a heterodimer with another bHLH protein that could be necessary for cell proliferation of the wing disc, and consequent titration of this protein (a dominant negative effect). However, the high level of

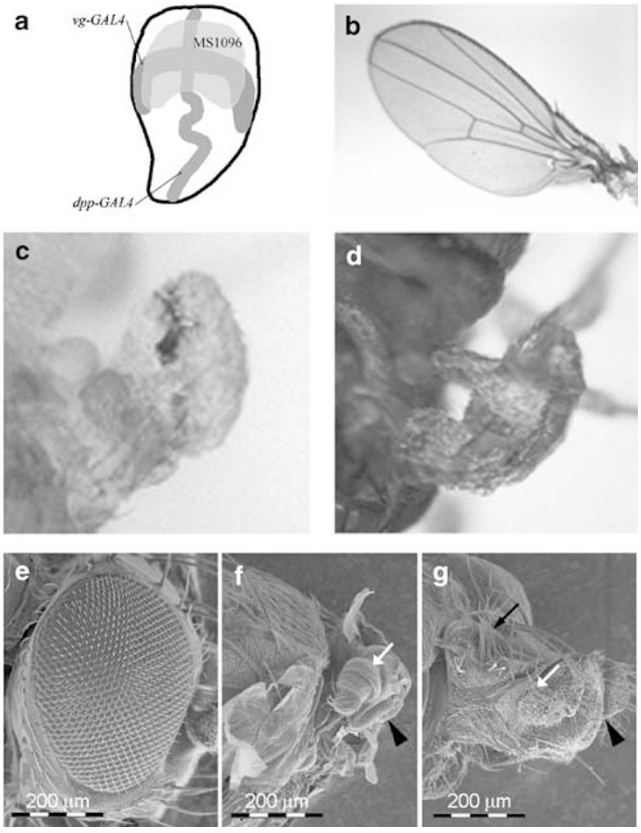


Figure 2 (**a**) Schematic representation of the different GAL4 drivers used to study *twist* ectopic expression in the wing disc. The *MS1096-GAL4* driver is expressed throughout the wing pouch. It corresponds to an insertion in the *Beadex* locus. The *vg-GAL4* driver allows expression according to the *vg* wing boundary enhancer, with a high level in the part of the disc that will give the future wing margin. The *dpp-GAL4* driver allows expression in the anterior compartment of the wing disc at the level of the boundary between the anteroposterior compartments. (**b**) Wing of an adult wild-type strain. (**c**) Wing of a F1 from the cross between *UAS-twistD* and *MS1096-GAL4* flies. A strong reduction in wing size is observed. However, the wing margin is still present. (**d**) Wing of a F1 from the cross between *UAS-twistCE* and *MS1096-GAL4* flies. A similar phenotype to that in C is observed. (**e–g**) Electronic microscopy of an eye and the head of adult flies. Anterior is at the right side, and dorsal face at the top. Same scale was used for each genotype: (**e**) Eye of wild-type strain. (**f**) Head of a F1 from the cross between *UAS-twistD* and *eye-GAL4* flies. Complete disappearance of the eye is observed. Antenna (white arrow) and trunk (black arrow head) can be identified, but other head structures are absent. (**g**) Eye of a F1 from the cross between *UAS-twistCE* and *eye-GAL4* flies. Antenna (white arrow) and trunk (black arrow head) can be identified. A strong reduction of the eye, identified by the specific bristles, is observed (black arrow)

expression of the transgenes and the specificity of the dimerization between the bHLH proteins argue in favor of the former hypothesis.[22]

Ectopic expression of the *Drosophila* transgene induces apoptosis

In order to understand the wing phenotype observed upon ectopic expression of the *Drosophila twist* transgene, we examined apoptosis in wing imaginal discs by staining with acridine orange. Using the *MS1096-GAL4* driver, we observed a significant amount of apoptosis in the wing pouch where this driver is most strongly expressed, compared to the control (Figure 3a,b). This result was confirmed using the *vg-GAL4* driver (not shown).

To understand how the *twistD* transgene induces apoptosis, we tested for possible induction of the *reaper* cell death gene. We ectopically expressed the *Drosophila twist* transgene using a *decapentaplegic-GAL4 (dpp-GAL4*), *reaper-LacZ* strain, where *LacZ* is under the control of the *reaper* promoter. Wing discs were stained with both anti-β-galactosidase antibody and *Drosophila* TWIST antibody. Strong induction of *reaper* was observed along the length of band of the driver expression, confirming that the transgene induces apoptosis by activating *reaper* (Figure 3c–f). However, induction of *reaper* by *Drosophila twist* extends beyond the domain of expression of *dpp*, showing that the effect on *reaper* is noncell autonomous, and suggesting that *reaper* induction is not a direct effect of *twist* on the *reaper* promoter. The effect of ectopic expression of *twist* seems to be restricted to the tissue where *twist* is not normally expressed. Indeed, no induction of apoptosis was observed at third larval instar when the expression of *twistD* is directed to the myoblasts located on wing imaginal discs using the *1151-GAL4* driver (not shown). However, defects in adult thoracic muscles have been reported to occur when *Drosophila twist* is expressed in the wing disc myoblasts, even if the cause of these defects has not been identified yet.[23]

The same experiments were performed with the *UAS-twistCE* transgene, but no apoptosis was observed when the transgene was ectopically expressed in wing imaginal discs using either the *MS1096-GAL4* or the *vg-GAL4* driver (data not shown). No activation of the *reaper* promoter was observed either (data not shown). These results exclude the possibility that the abnormal wing phenotype observed in this case was because of apoptosis. No apoptosis was observed using the *UAS-twistH* transgene either (data not shown).

Ectopic expression of the nematode transgene induces arrest of proliferation

To explain the wing phenotype because of the ectopic expression of the *C. elegans twist* transgene using the *MS1096-GAL4* driver, we hypothesized that the phenotype could be caused by a decrease in the proliferation rate.

To test this possibility, we compared the effect of overexpression of *dacapo* with that of overexpression of both *dacapo* and *twistCE* on wing development. *dacapo* is the homolog of human p21, which inhibits cyclin–cdk complexes and thus proliferation, yet does not induce apoptosis.[24] We used the *vg-GAL4* driver, which allows expression at the level of the wing margin. Overexpression of *dacapo* using this driver causes nicks at wing tips because of absence of proliferation.[25] Ectopic expression of *UAS-twistCE*, *UAS-dacapo* gives a stronger mutant phenotype than the ectopic expression of *UAS-dacapo*, *UAS-LacZ* (Figure 4a) suggesting that, like the *dacapo* gene, the ectopic expression of *twistCE* can affect proliferation. The *UAS-dacapo, UAS-LacZ* strain was used as a control, to exclude the possibility that the result is because of dilution of the GAL4 protein with an increased number of UAS transgenes.

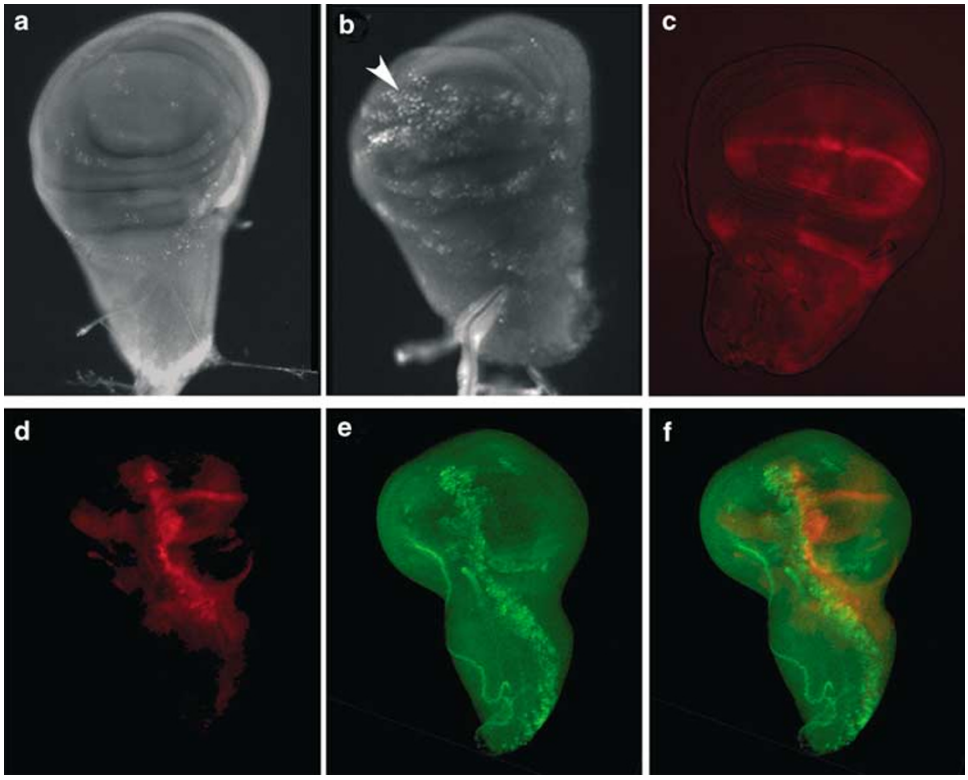


Figure 3 (**a**) Staining with acridine orange of a wild-type wing disc; no apoptosis is observed. (**b**) Staining with acridine orange of the wing disc of a F1 from the cross between *UAS-twistD* and MS*1096-GAL4* flies. Apoptosis in the wing pouch where the driver is expressed is observed (white arrow head). (**c**) *reaper-LacZ* expression in a third instar wing disc of a wild-type strain using anti-β-gal antibody. Endogenous expression of *reaper* occurs in the wild-type strain mainly at the level of the future wing margin. (**d**) *reaper-LacZ* expression in the wing disc of a F1 from the cross between *UAS-twistD* and *dpp-GAL4, reaper-LacZ* flies, using anti-β-gal antibody. Increased *reaper* expression according to the driver is observed. (**e**) The same disc stained with anti-TWIST antibody. (**f**) Merge of (**d**) and (**e**). Only a slight overlapping is observed and the domain of expression of *reaper* is larger than that of *twistD*, suggesting a non-cell autonomous effect

Another way to evaluate a possible role of *twistCE* in proliferation was to express the *UAS-twistCE* transgene ectopically in a genetic context that affects cell proliferation. The vg^{null} mutant phenotype is because of absence of proliferation at the wing margin. No apoptosis can be detected in wing imaginal discs of vg^{null} mutants and overexpression of *dacapo* using *vg* regulatory sequences in a vg^{null} heterozygous background enhances the effects of *dacapo*, suggesting synergistic effects between the function of the two genes in cell proliferation.[25]

We ectopically expressed *twistCE* under the control of *vg-GAL4*, and compared the effects when the *UAS-twistCE* strain was crossed with the *vg-GAL4,* vg^{null} strain. The vg^{null}/vg^{+} heterozygotes display a wild-type phenotype. The ectopic expression of *twistCE* using the *vg-GAL4* driver, in a vg^{null} heterozygous background dramatically enhanced the phenotype observed in a vg^{+} background (Figure 4b,c). This result strongly supports the hypothesis that the wing phenotype observed when *twistCE* is expressed in the wing disc is because of a significant decrease in cell proliferation.

The effect of *twistCE* on cell proliferation was further analyzed by *in situ* staining using anti-BrdU antibody that stains S phase cells (not shown) and by anti-phospho-histone H3 antibody that stains M phase cells (Figure 4d,e). When the *twistCE* transgene was overexpressed in either the wing or the eye imaginal discs (*MS1096-GAL4* and *eye-GAL4* drivers, respectively), no significant difference in BrdU staining was observed compared to the control indicating that the level of S phase cells was not sufficiantly altered to be detected by this method.

To evaluate an effect on mitotic cells, we then compared the number of M phase cells in the area defined by *MS1096* expression, by double staining with anti-phospho-histone H3 and anti-β-galactosidase antibodies, with or without *twistCE* ectopic expresssion. We used this driver, as it allows expression in the whole wing pouch, to increase statistical significance. The wing pouch of 10 discs from the progeny of each cross were counted, and we found an average of 47 M phase cells per disc for the control and only 18 M phase cells per disc when *twistCE* was ectopically expressed (65% reduction of M phase cells), confirming the role of the *twistCE* transgene on the rate of mitosis. This effect was restricted to the area where the driver is expressed. For instance, in the notum, there was no difference between the control cross and the flies expressing *twistCE*. Indeed, an average of 147 M phase cells per notum were found for the Canton wild-type strain, and 141 M phase cells per notum for the *twistCE* transgene expressing flies on 10 wing discs tested for each strain.

a

Fly genotype \ Wing phenotype	wild type	nicked wing	notched wing	wing stump
vgGAL4	100	0	0	0
vgGAL4, UASdap, UASlacZ	86.11	13.89	0	0
vgGAL4, UAStwistCe, UASlacZ	0	3.46	91.15	5.39
vgGAL4, UASdap, UAStwistCe	0	0.53	86.77	12.70

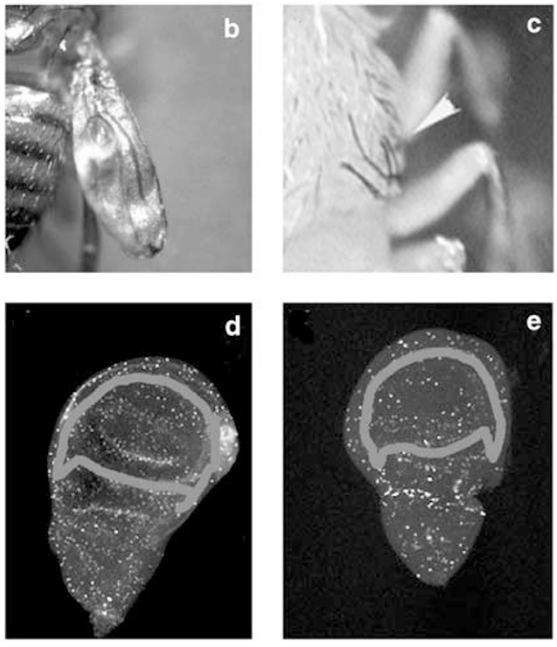


Figure 4 (**a**) Different wing phenotypes observed when *dacapo*, *twistCE* and both *dacapo* and *twistCE* are ectopically expressed in the wing disc using the *vg-GAL4* driver. The *UAS-LacZ* transgene was used to control for the possibility that the differences observed are because of dilution effects. In all, 500 wings were scored for each cross. (**b**)Wing of a F1 from the cross between *UAS-twistCE* and *vg-GAL4* flies. A scalloped wing phenotype is observed. (**c**) Wing of a F1 from the cross between *UAS-twistCE* and *vg-GAL4, vg*^{null} flies. In this heterozygous *vg*^{null} background, a complete absence of the wing is observed (arrow for the normal place of the wing). (**d**) Anti-phospho-histone H3 staining of a third instar wing disc from the *MS1096-GAL4* strain. The antibody stains cells in M phase (in red the limits of driver expression). (**e**) Anti-phospho-histone H3 staining of a third instar wing disc from the cross between *UAS-twistCE* and *MS1096-GAL4* flies. A decrease in the number of M phase cells is observed according to the driver. We estimated the decrease in M phase in the wing pouch to be an average of 65%

The fact that the transgene displays no effect using anti-BrdU staining suggests a specific effect on the G2/M transition. A similar effect, but less spectacular, was observed using the *1151*-GAL4 driver that allows expression in the myoblasts located on the notum of the wing imaginal disc (not shown).

Finally, the two other *twist* transgenes (*UAS-twistD* and *UAS-twistH*) did not decrease the number of M phase cells (data not shown). This indicates specificity in the effects of the different TWIST proteins: induction of apoptosis for *twistD* and a decrease in the mitotic rate for *twistCE*.

Ectopic expression of human *twist* inhibits p53-dependent apoptosis

Ectopic expression of human *twist* did not induce a wing phenotype. In mammals, it has been reported that *twist* can be considered a proto-oncogene that acts by inhibiting p53-dependent apoptosis.[19]

Conservation in *Drosophila* of the function of human *twist* would indicate conservation between the human and *Drosophila* p53 pathways and that human *twist* cooperates with the equivalent *Drosophila* components of the pathway, and activates *Drosophila* targets that are homologs of human p53 targets.

In *Drosophila*, it has been shown that p53 activates the *reaper* promoter but is unable to stop proliferation, and has no effect on *dacapo*, the p21 homolog.[26,27]

In order to test the effects of human *twist* on apoptosis, we decided to compare the effects of the *twistH* transgene to the effects of a transgene encoding a dominant-negative form of *Drosophila p53*. This transgene (called here *UAS-p53**) has been shown to inhibit apoptosis induced by ionizing radiation.[26,27]

To facilitate the evaluation of the decrease in cell death induced by the dominant-negative *UAS-p53** or by the *UAS-twistH* transgene, we decided to express the transgenes in the wing disc and discriminate the effects on the change in wing phenotype compared to a control.

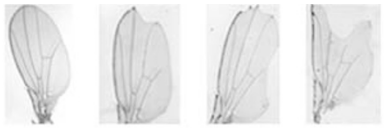
However, in the wing disc, massive cell death does not automatically lead to a wing phenotype, and only death of cells that will give the future wing margin affects wing morphology. Indeed, no adult wing phenotype were observed after exposure of larvae to ionizing radiation or UV, although acridine orange staining was clearly positive (data not shown).

Aminopterin, an analog of methotrexate, induces massive cell death and a well characterized wing phenotype when cell death occurs in the part of the wing disc that will give the future wing margin.[28] This drug is an inhibitor of dihydrofolate reductase activity, and therefore inhibits DNA replication and the G1/S transition.

We first tested whether cell death induced by aminopterin is p53 dependent by using the dominant-negative *p53** to try to inhibit the effect of the drug. When the *UAS-p53** transgene is ectopically expressed using the *vg-GAL4* driver, no wing phenotype is observed. On a concentration of aminopterin (2 mg/kg) that induces nicks in the wings with the *vg-GAL4* strain that serves as control, the transgene counteracts to some extent the effect of the drug (51.4% notched wings, n=600 for the control *versus* 40.2% notched wings, n=616 for p53*-expressing flies).

To enhance the effects of the aminopterin, we used a heterozygous *vg*^{null} mutant context that is completely recessive on normal medium, but induces a high proportion of notched wings on a low concentration of aminopterin (0.5 mg/kg) (Figure 5a). As expected, the effect of the *UAS-p53** transgene is more spectacular in a *vg-GAL4; vg*^{null}*/vg*$^{+}$ genotype (Figure 5a) and significant differences were

a



Fly genotype \ Wing phenotype	wild type	nicked wing	notched wing	extreme phenotype
vgGAL4, vg^{null} / +	0	0	50.8	49.2
vgGAL4, UASp53, vg^{null} / +*	0	0	100	0
vgGAL4, UAStwistH, vg^{null} / +	9.3	26.2	60.8	3.7

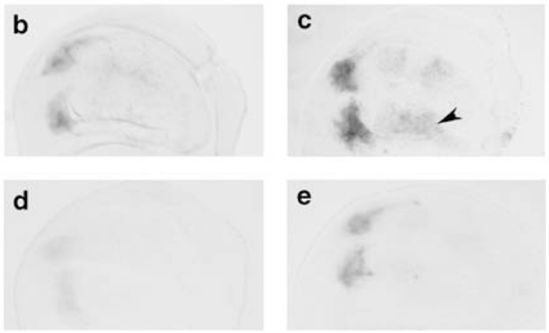


Figure 5 (**a**) The *human twist* transgene restores the wing phenotype from the effects of aminopterin. Proportion of the different wing phenotypes observed when *+/vg^{null}* flies, carrying different transgenes combinations, are reared on aminopterin (1 mg/kg). In the control (*vg-GAL4, +/vg^{null}*), half of the flies have 'notched' wings and half of a more 'extreme' phenotype when reared on aminopterin. Overexpression of *UAS-p53** using *vgGAL4* sequences shifts the phenotype of *vg^{null}/+* heterozygotes from the effect of aminopterin towards only the 'notched' wings phenotype. The effect of the *twistH* transgene on *vg^{null}* heterozygotes is more pronounced than with the *UAS-p53** transgene, since 'nicked' wing and 'wild-type' wing phenotypes appear. (**b**) Expression of the 150 bp *reaper-LacZ* construction in the wing disc of third instar larvae on normal medium. The same condition for *β*-gal staining were applied for **b–e**. (**c**) Expression of the 150 bp *reaper-LacZ* in the wing disc of third instar larvae reared on aminopterin (1 mg/kg). An increase in staining is observed. (**d**) Effect of overexpression of *UAS-p53** using the *MS1096-GAL4* driver on the 150 bp *reaper-LacZ* expression in the wing disc of third instar larvae reared on aminopterin (1 mg/kg). A decrease in the intensity of the staining is observed. (**e**) Effect of overexpression of *UAS-twistH* using the *MS1096-GAL4* driver on the 150 bp *reaper-LacZ* expression in the wing disc of third instar larvae reared on aminopterin (1 mg/kg). As for *UAS-p53** expression, compared to C, a decrease in the staining is observed

observed when compared to the control (*vg-GAL4; vg^{null}/vg^{+}* strain). These results indicate that apoptosis induced by aminopterin is p53 dependent, and that this drug can be used to evaluate p53 activity.

The *UAS-twistH* transgene was also ectopically expressed using the *vg-GAL4* driver first in a *vg^{+}* context. The F1 was reared on aminopterin (2 mg/kg), and we observed 42% notched wings (n=376) compared to *vg-GAL4; vg^{+}/vg^{+}* flies that served as control (51.4%, n=600). These results show that, as with the *UAS-p53** transgene, *UAS-TwistH* reduced to some extent the effect of aminopterin.

Next, the *UAS-twistH* transgene was expressed using the *vg-GAL4* driver in a *vg^{null}* heterozygous context. The F1 was reared on aminopterin (0.5 mg/kg) and the wing phenotype compared to that of *vg-GAL4; vg^{null}/vg^{+}* flies that served as a control for the experiment with the *UAS-p53** transgene. As for *UAS-p53**, a significant difference was observed compared to the control (Figure 5a), suggesting that the human *twist* transgene shares properties with *UAS-p53**. In fact, the *UAS-twistH* transgene is significantly more potent, shifting the wings more towards a wild-type phenotype than *UAS-p53** (Figure 5a). The effect observed is not because of the genetic *vg* context as it was observed in both a *vg^{+}* and a *vg^{null}* heterozygous backgrounds.

The next step was to determine whether the effect of *UAS-twistH* is p53 dependent. p53-dependent apoptosis is because of the direct binding of p53 to the *reaper* promoter. It has been shown that a sequence of 150 bp in the *reaper* promoter responds specifically to activation by p53.[26] We observed that a transgene that will be called here 150 bp *reaper-LacZ* (a gift of J Abrams) is activated by p53 in the wing imaginal discs when flies are reared on aminopterin (Figure 5b,c). In the wing disc of the F1 of the cross between *UAS-p53** and *MS1096-GAL4,* 150 bp *reaper-LacZ* flies raised on aminopterin, as expected, a significant diminution of X-Gal staining was observed in the wing pouch (Figure 5d). This confirms that the dominant-negative form of p53* disrupts activation of *reaper* transcription by active endogenous p53 via the 150 bp sequence from the *reaper* promoter.

The same cross was done using the *UAS-twistH* transgene instead of *UAS-p53** with the same result, indicating that the TWISTH product counteracts the active wild-type *Drosophila* p53 (Figure 5e). However, we cannot exclude the possibility that repression of the 150 bp *reaper-LacZ* transgene by *twistH* is not due to the effect on p53 but rather to the effect on other factors that bind this *reaper* regulatory element. Another *LacZ* strain, called the *p53 response element* has been described, which consists of oligomers of the p53 binding sites in the *reaper* promoter region.[26] However, it cannot be used in the wing disc as it is not activated in this tissue by p53 (data not shown). The possibility that the *Drosophila* and nematode transgenes induce a similar effect was tested without positive results, indicating that the results were specific to the human transgene.

Taken together, these results strongly suggest that human *twist*, expressed in *Drosophila*, retains some of the same properties it has in human cells, in particular inhibition of p53-dependent apoptosis. Moreover, in *Drosophila*, it acts on the *reaper* promoter.

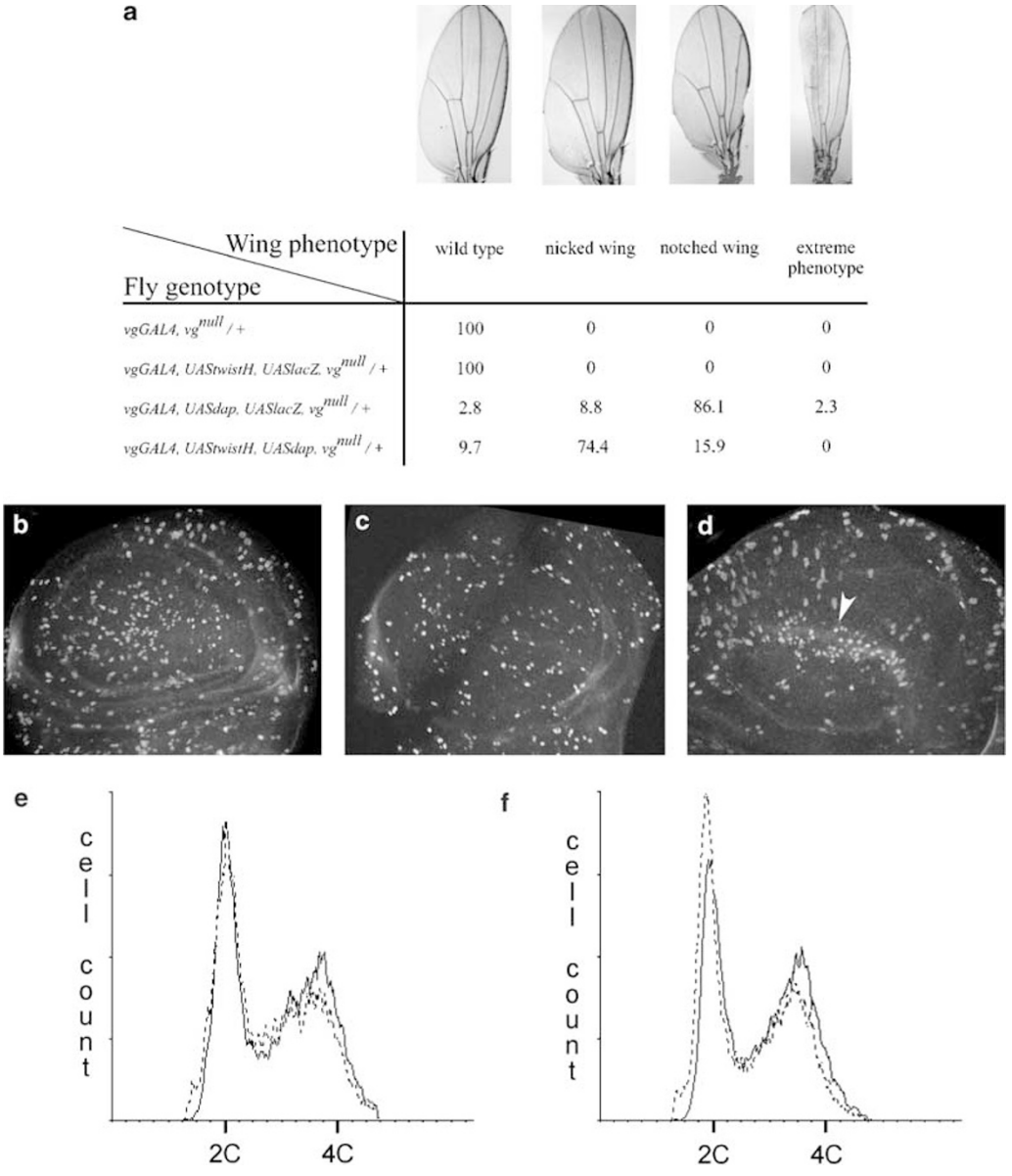
Ectopic expression of human *twist* induces cell proliferation

It has been shown that vertebrate *twist* is able to induce proliferation of skeletal muscle satellite cells.[29] However, a clear role for vertebrate *twist* in proliferation has not been well established. Using our heterologous system, we tested if the human *twist* transgene is able to induce proliferation in *Drosophila*.

For this, we compared the wing phenotypes induced in the F1 of the cross between *UAS-dacapo, UAS-LacZ*, and *vg-*

GAL4, vg^{null} flies to those in the F1 of the cross between *UAS-dacapo, UAS-twistH*, and *vg-GAL4, vg*^{null} flies. In this *vg*^{null} heterozygous background, nicks in the wing were observed in the former cross (Figure 6a), and this was not due to apoptosis but to inhibition of cell proliferation.[24] In the latter cross, a significant rescue of the wing phenotype was observed, indicating that human *twist* can partially supress the *dacapo* overexpression phenotype, and thus increases cell proliferation and/or cell growth.

To confirm this result, we stained the wing disc with anti-phospho-histone H3 antibody that marks cells in mitosis. An increase in staining was observed when human *twist* was expressed along with *dacapo* (*UAS-twistH, UAS-dacapo*) using the *vg-GAL4* driver compared to the control (*UAS-*



Fly genotype \ Wing phenotype	wild type	nicked wing	notched wing	extreme phenotype
vgGAL4, vg^{null} / +	100	0	0	0
vgGAL4, UAStwistH, UASlacZ, vg^{null} / +	100	0	0	0
vgGAL4, UASdap, UASlacZ, vg^{null} / +	2.8	8.8	86.1	2.3
vgGAL4, UAStwistH, UASdap, vg^{null} / +	9.7	74.4	15.9	0

Figure 6 (**a**) The *twistH* transgene is an antagonist of the overexpression of *dacapo*. In the control *vg-GAL4, vg*^{null}/+ flies or when *twistH* is overexpressed using the *vg-GAL4*, only a wild-type wing phenotype is observed. Ectopic expression of *dacapo* or *twistH* and both *dacapo* and *twistH* in the wing disc using the *vg-GAL4* driver in a *vg*^{null} heterozygous background was assayed. Coexpression of *dacapo* and *twistH* restores the wing phenotype induced by *dacapo* towards a 'nicked' wing phenotype instead of a 'notched' phenotype. (**b**) Anti-phospho-histone H3 staining of cells in M phase in a third instar wing imaginal disc of the *vg*^{null} heterozygous control strain. (**c**) Anti- phospho-histone H3 staining of a third instar wing imaginal disc of a F1 from the cross between *UAS-dacapo* and *vg-GAL4* flies, in a *vg*^{null}/+ background. A decrease in staining in the wing pouch is observed. (**d**) Anti- phospho-histone H3 staining of a third instar wing imaginal disc of a F1 from the cross between *UAS-dacapo, UAS-twistH* and *vg-GAL4* flies, in a *vg*^{null}/+ background. An increase in cell undergoing mitosis is observed where the driver is expressed, compared to C (arrow head). (**e–f**) Flow cytometry. Uninterrupted and dotted lines represent GFP^{+} (transgene-expressing posterior compartment) and GFP^{-} (control anterior compartment) cells, respectively. Each trace is normalized to fit the graph as the number of GFP^{+} and GFP^{-} cells analyzed for each sample were not exactly equal. (**e**) Control: cells from third instar larvae wing imaginal discs from the cross between *UAS-lacZ* and *en-GAL4, UAS-GFP* flies. (**f**) Overexpression of the human *twist* transgene: cells from third instar larvae wing imaginal discs from the cross between *UAS-twistH* and *en-GAL4, UAS-GFP* flies

dacapo, UAS-LacZ) showing that human *twist* drives cells in mitosis (Figure 6b–d).

To evaluate the rate of cell proliferation, we used the fluorescence-activated cell sorter (FACS). We performed FACS analysis using an *en-GAL4; UAS-GFP* line. This strain expresses the green fluorescent protein (GFP) under control of *engrailed* regulatory sequences in the posterior compartment of the wing disc, and so allows us to distinguish between wild-type cells and transgene-expressing cells.[30] We compared the F1 from the cross between *UAS-LacZ* and *en-GAL4; UAS-GFP* flies, which serves as an external control, to the F1 from the cross between *UAS-twistH* and *en-GAL4; UAS-GFP* flies. We used wing disc from larvae precisely staged at 120 h AED (after egg deposit) to measure the proportion of cells in G1, S and G2/M phases in the two crosses. We compared cells from the posterior compartment (GFP^+, transgene-overexpressing compartment) to cells from the anterior compartment (GFP^-, internal control compartment). Two sets of experiments were performed independantly, and data were analyzed using the Multicycle software. The control cross confirms that there is no significant difference between the two compartments because of the expression of a transgene in only one of the two compartments (Figure 6e). Indeed, the mean percentage change of GFP^+ cells compared with GFP^- cells in the proportion of cells in G1 phase is a decrease of 3.05% for control experiments (Figure 6e, Table 1), which is not statistically significant. Conversely, the mean percentage change of cells in G1 is of 9.65% for *UAS-twistH* overexpression experiments (Figure 6f, Table 1), which is significant ($P<0.01$). Moreover, the mean decrease in the proportion of G1 cells in the control cross is significantly different from that in the *twistH*-overexpressing cross ($P<0.1$), and the mean increase of G2/M cells proportion is also significantly different ($P<0.1$) (Table 1). However, no significant difference can be observed considering the proportion of S phase cells. Those results indicate that the *twistH* transgene causes a shift from the G1 phase to the G2/M phase of cell cycle, and so is an activator of cell proliferation.

Furthermore, the analysis of the Forward Scafold data, which is a measure of cell size, reveals no difference in average cell size between the two compartments in overexpression experiments (data not shown). This is surprising as overproliferation is generally accompanied by a reduction in cell size. But in fact, this could be explained if TWISTH not only activates cell proliferation but also promotes cell growth.

Table 1 Table shows the mean percentages in two experiments, for GFP^- control cells and GFP^+ transgene-expressing cells, and the mean changes between GFP^- and GFP^+ cells

	Mean percentage				**Mean change**	
	GFP^-		**GFP^+**			
	G1	**G2/M**	**G1**	**G2/M**	**ΔG1**	**ΔG2**
Control	33.15	34.35	30.1	36.7	−3.05	+2.35
Human *twist* overexpression	35.55	32.55	25.9	48.3	−9.65	+15.75

These data indicate that human *twist* retains, in *Drosophila*, the proto-oncogenic properties that have been described in vertebrate cultured cells, decreasing p53-dependent cell death and inducing proliferation. Even if the p53 pathways are significantly different between human and *Drosophila*, the human TWIST protein retains its potentials when expressed in the fly thus further supporting its role as an inducer of cell proliferation, *per se*, even in an epithelial tissue like the wing disc.

Discussion

Numerous transgenic *Drosophila* strains have been engineered to express human gene to better understand their function (for a review, see Chan and Bonini[21] and Link[31]). This has been the case, in particular, for genes involved in neurodegenerative diseases like Alzheimer's,[32,33] Parkinson,[34] polyglutamine disease,[35] and amyotrophic lateral sclerosis.[36] Thus, we used this method to study the potentialities of human TWIST and unravel new putative functions of the TWIST proteins of other species. In addition, this approach provides a useful tool to isolate modifiers of the different phenotypes induced by the ectopic genes.

Ectopic expression of *Drosophila twist* induced apoptosis and activated *reaper* expression. In contrast, ectopic expression of *twistCE* in the same cells did not induce apoptosis, but an arrest of proliferation that mimics the effect of *dacapo*, the homolog of p21.

The induction of apoptosis by *twistD* in epithelial cells cannot be considered only as the result of an overload of ectopic protein, as neither *twistCE* nor *twistH* induce apoptosis. We showed that *twistD* has the potentiality to activate the proapoptotic pathway in *Drosophila*, clearly at least *repear* expression. The effect seems to be noncell autonomous, suggesting that it is not a direct effect on the *reaper* promoter as has been shown for *Deformed*, another developmental gene.[37] The question now is if this potentiality of *twistD* is used during development or if it is only because of an ectopic effect. The fact that *twist* is expressed in proliferating myoblasts is an argument for a possible role in apoptosis in deregulated conditions. This could be linked to the observation of degeneration of the indirect flight muscles in *twist* hypomorph or when *twist* is overexpressed in the myoblasts. It has been suggested that degeneration is caused by apoptosis.[23] This point will require further investigation.

twistH is able to act on the apoptotic pathway by repressing p53-dependent *reaper* activation. We provide several lines of evidence indicating that *twistH* is a suppressor of p53-dependent apoptosis: (i) its ectopic expression induces the same effect as that of a dominant negative form of *p53** and (ii) it represses the 150 bp *reaper* regulatory element that is normally activated by wild-type p53. This suggests that ectopic expression of *twistH* perturbs *Drosophila* p53 activation of some of its natural targets, at least *reaper*. This indicates that the potentialities of the TWIST protein are not restricted to one cell type and that the capacity for human TWIST to inhibit p53-dependent apoptosis can occur *in vivo* as we were able to reproduce it in *Drosophila*. The capacity to repress the apoptotic pathway in human cells has also been

described for *DermoD*, which presents extensive homology with *twistH* principally at the level of the bHLH domain. This suggests that this domain is implicated in the phenomenon.[19]

However, here the effect observed is on *Drosophila* p53. It has been reported that if *Drosophila* p53 shares with mammalian p53 the capacity to respond to DNA damage via induction of apoptosis, it seems unable to induce a G1 arrest by activating *dacapo*, the *Drosophila* p21 homolog.[26,27]

Another important difference between *Drosophila* and mammalian p53 is that the residues critical for MDM2 binding are not conserved in *Drosophila* p53.[27] MDM2 is a potent negative regulator of p53 that acts by binding to, and thus inhibiting, the p53 transactivation domain and by fostering p53 degradation.[38,39] Stabilization of p53 occurs when the ARF tumor suppressor interferes with MDM2 function.[40] ARF binds directly to MDM2 thus enabling transcriptionally active p53 to accumulate in the nucleoplasm.[41,42]

It has been suggested that the effects of the ectopic expression of *twistH* on p53-dependent apoptosis in cultured cells are mediated by the effects of *twist* on ARF.[19] Indeed, transient expression of *twistH* induces a dramatic down-regulation of ARF, thus providing a potential mechanism by which *twist* may affect p53 function. However, since *reaper* is the only identified target of *Drosophila* p53 at the moment, it is difficult to explore further how *twistH* could directly counteract *Drosophila* p53.

Our results indicate conservation of *twistH* potentialities in *Drosophila*. This suggests that either: (i) in fact, the ARF/MDM2/p53 pathway exists in *Drosophila* but ARF and MDM2 have not been identified yet or (ii) *twistH* acts on *Drosophila* p53 activity by affecting an alternative pathway. Indeed, *twistH* has been shown to interact *in vitro* with P300/CBP, regulating its histone acetyltransferase activity, and thus could interfere with p53 activity altering its ability to bind target sequences *in vitro*.[43]

Moreover, recently, it was shown that the antiapoptotic action of insulin-like growth factor (IGF-1) signaling is mediated by induction of *twist* suggesting a key role for Mammalian *twist* as an antiapoptotic factor.[20] Thus, a link between the antiapoptotic action of the IGF-1 receptor and p53-dependent apoptosis via *twist* can be suggested.

In this study, we also considered Twist potentialities on cell proliferation. We showed that both *twistCE* and *twistH* could act on proliferation. Ectopic expression of *twistCE* induces an arrest of proliferation. This was clearly observed using an anti-phospho-histone H3 antibody that stains mitotic cells, while no effect was observed with anti-BrdU antibody that marks cells in S phase suggesting the possibility that the G2/M transition is principally affected. In addition, ectopic expression of *twistCE* aggravated the reduced wing phenotype induced by *dacapo* and by a decrease in VG protein dose, confirming that *TwistCE* can antagonize the normal mitotic process. The fact that *TwistCE* is expressed only in differentiated mesodermic tissue can perhaps be linked to our results.[11]

In contrast, *twistH* is able to increase epithelial cell proliferation in *Drosophila*. FACs analysis showed that ectopic expression of human *twist* shifts the repartition of the cells toward a decrease in the percentage of cells in G1 to an increase in the percentage of cells G2/M, suggesting an effect of TWISTH principally on mitosis. Such a proliferative function has not been clearly reported so far in mammalian cultured cells or in *in vivo* system. However, it has been shown that hepatocyte growth factor (HGF) inhibits skeletal muscle cell differentiation and stimulates cell proliferation of satellite cells probably by inducing *twist* expression.[29] Interestingly, IGF-1 can also promote proliferation of satellite cells,[44] thus linking the role of vertebrate *twist* in p53-dependent apoptosis and proliferation.

On the whole, the transgenic strains expressing ectopic *twist* enable us to establish different potentialities for *twistD* and *twistCE* that can now be investigated to determine whether *twistD* induction of apoptosis and *twistCE* inhibition of mitotic activity occur naturally during the developmental processes where *twist* is required. To identify which domains of the TWIST protein are responsible for the phenotypes observed, chimeric transgenic lines where bHLH domain and/or the WR motif of one species would be replaced by that of another should prove informative. Other domains of the TWIST protein can also be analyzed in this way.

Most important, we show that the role of *twistH* as a potential oncogene can be studied *in vivo* using *Drosophila* transgenic lines suggesting that the targets of p53-dependent apoptosis and induction of proliferation are conserved in *Drosophila*. This gives us a powerful tool to screen for targets and for partners of the p53 and proliferation pathways, as well as modifiers.

Materials and Methods

Generation of trangenics flies

UAS-twistH and *UAS-twistCE* strains were constructed in our laboratory by transgenesis.[45] The *twist* cDNAs, gifts from Fabienne Perrin-Shmid[46] and Andrew Fire,[10] respectivily, were cloned into pCAsPeR vector.[47] Five independent lines were obtained with the human transgene, and three with the nematode transgene. For both transgenes, all lines give a similar result.

Fly stocks

The *Drosophila UAS-twist* line (*UAS-twistD*) was provided by Mary Baylies.[7] *UAS-p53** was a gift from Dr. Kopczynski.[27]

The different GAL4 drivers used were obtained from the Bloomington stock center. *GMR-GAL4* expression is restricted to the presumptive photoreceptor cells posterior to the morphogenetic furrow of the eye imaginal disc, whereas *eye-GAL4* is expressed in the entire eye-antenna imaginal disc, and thus usually leads to more drastic effects. The *dpp-GAL4* strain (Figure 2a) was used to drive expression in both eye imaginal discs, specifically in the morphogenetic furrow, and wing imaginal discs, specifically at the anteroposterior compartment boundary. The *vg-GAL4* driver, which is expressed at the dorso-ventral compartment boundary (Figure 2a), allows us to express the transgenes at the level of the future wing margin that is very sensitive to a decrease in cell number, and thus can induce an easily visible wing phenotype. The *MS1096-GAL4* driver (Figure 2a), which drives expression in the entire wing pouch, and also displays a faint and variable expression in the notum, was used to conduct statistical experiments on proliferation rates in the wing pouch, as it gives a larger expression domain than other wing drivers. The *1151-*

GAL4 driver allows expression in the myoblasts located on the notum of the wing disc.

The reporter construct strains were provided by Dr. Abrams.[26] The *reaper-LacZ* strain carries the *LacZ* sequences under the control of the entire promoter of the *reaper* gene; the 150 bp *reaper-LacZ* reports the activation of the 150 bp of the *reaper* promoter that respond to the *Drosophila* p53 protein.

We also used the *vestigial* null mutant, named vg^{null}. The vg^{null} mutant leads to absence of proliferation at the wing margin, and displays complete absence of wings in homozygous adults.[48]

Acridine orange staining

Detection of apoptotic cells was performed with acridine orange staining. Third instar larvae were dissected, and imaginal discs were collected in PBS 1 $\times$. Imaginal discs were then incubated 2 min in a 0.6 μg/ml[1] acridine orange/PBS 1 $\times$ solution and rinsed very briefly in PBS 1 $\times$ before observation.

Histochemical detection

LacZ staining was performed on dissected wing imaginal disc. Third instar larvae were dissected in PBS 1 $\times$, fixed in 4% formaldehyde–1% glutaraldehyde and wing discs were incubated in a 0.04% X-gal solution overnight. All genotypes were treated simultaneously to avoid variation because of different experiments.

Immunostaining was performed as previously described.[25] Antibody against *Drosophila* TWIST protein was a gift from S Roth[49] and was used at 1/5000 dilution. Antibody against human TWIST protein is avalaible at Santa Cruz Biotechnology and used at 1/200 concentration. Anti-phospho-histone H3 stains specifically phosphorylated histone 3, and so allows detection of cells in mitosis; anti-phospho-histone H3 antibody is available at Upstate Biotechnology used at 1/2000 concentration. Anti-β-gal antibody (from Amersham) is a mouse antibody and was used at 1/200 dilution.

Fluorescence-activated cell sorter (FACS) experiments

Staged larvae of 120 h AED, derived from 2 to 3 h egg collections, and raised at 25°C were dissected in PBS. In all, 20 wing discs were washed twice in PBS and incubated with gentle agitation for 150 min according to Neumann and Edgar.[50] An Elite Beckman Coulter FACS was used, and data were analyzed using the Multicycle Software.

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