The regulated expression of an intrabody produces a mutant phenotype in *Drosophila*

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Abstract Intrabodies show great promise for controlling gene expression. As an initial attempt to evaluate the intrabody technology in *Drosophila*, the gene *poxn* was used as target. Transgenic flies harboring different anti-Poxn scFv genes integrated into various chromosomes were obtained. In one transformant, a phenocopy resembling the hypomorphic *poxn*$^{-}$ phenotype was produced in embryos and larvae following induction of expression of α-Poxn2 intrabody. The antisense approach was used as control. Parameters that can affect the success of intrabody technology are described.

Key words: *Poxn*; Phenocopy; Intrabody; Antisense; *Drosophila*

1. Introduction

Intrabody technology, the intracellular expression of antibodies, is a promising new approach to manipulate biological processes. Cell culture assays have clearly established that intrabodies are able to interfere in a specific manner with protein and non-protein molecules and even with distinct pools of the same protein acting in distinct cellular compartments (reviewed in [1,2]).

The promising successes with intrabodies in cell cultures have been followed by very few experiments at the whole organism level (for examples see [3,4]). This is partly because a convenient assay system has not yet been introduced.

Several features make *Drosophila* an ideal system for assessing the intrabody technology in a whole organism (see accompanying paper [34]).

In this study, we address the feasibility of intrabody-mediated modulation of neuronal development in *Drosophila*.

The embryonic and larval peripheral nervous system (PNS) in *Drosophila* comprises few different types of sense organs. Two types of external sense (es) organs are found on the first seven abdominal segments (A1–7): the papillae (p) and the sensory hairs (h). All papillae, except the abdominal papilla p6, are mono-innervated and probably function as strain receptors. All sensory hairs, except the abdominal hair h3, are also singly innervated and mediate touch [5,6]. The thoracic segments (T1–3) also bear Keilin's organs which are humidity receptors [7,8], and the peglike kölbchen [9] with as yet unknown function. Each Keilin's organ is a group of three hairs, innervated by five neurons [5,10]. The pattern of innervation of Keilin's organ is still a subject of debate (for example see [11]). All kölbchen are innervated by three neurons, except the dorsal T1 kölbchen which is innervated by two neurons. It has been demonstrated that the doubly innervated abdominal organs p6 and h3 are the abdominal homologues of the thoracic kölbchen [5,6].

Since each type of sense organ has a characteristic morphology and distribution on the body of the embryo and larva (for the map of embryonic and larval es organs, see [5]), it is easy to screen for phenotypic changes that reflect altered activities of genes controlling various sense organs. Among these control genes is the gene *poxn* (*paired box neuro*) which determines poly-innervated external sense (p-es) organs. *Poxn* is expressed in a few clusters of cells in the developing peripheral and central nervous system [11,12]. In the peripheral nervous system, the cells that express *poxn* will form the thoracic kölbchen and their abdominal homologues, p6 and h3. In the absence of *poxn*, the thoracic kölbchen and the abdominal papilla p6 are always missing, and there is a four-fold decrease in the number of abdominal hair h3. The ectopic expression of *poxn* results in the transformation of mono-innervated external sense (m-es) organs into poly-innervated external sense (p-es) organs, most easily detected by the formation of supernumerary kölbchen on the thoracic segments [11]. The role of *poxn* in the development of the central nervous system is not yet known.

In this paper, we report on the inhibition of *poxn* function by intrabodies. We demonstrate that intrabodies can be ubiquitously expressed throughout the life of the fly without causing non-specific toxic effects, and that intrabodies can cause detectable gene suppression in *Drosophila*. As a control, we used the antisense approach to produce a hypomorphic mutant phenotype.

2. Materials and methods

2.1. General molecular techniques

Unless otherwise noted, nucleic acids were handled according to standard protocols [13]. Reagents for PCR, PCR conditions, purification of nucleic acids from agarose gel, expression in *Escherichia coli* and purification of *poxn* protein and scFvs antibodies, determination of antibodies specificities and half maximal inhibition values were as in Hassanzadeh et al. (accompanying paper) except where mentioned.

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Abbreviations: CNS, central nervous system; es organ, external sense organ; m-es organ, mono-innervated external sense organ; PCR, polymerase chain reaction; p-es organ, poly-innervated external sense organ; PNS, peripheral nervous system; scFv, single-chain variable fragment

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2.2. Construction of hsp70-anti-poxn constructs

The scFv-c-myc tag fusion genes were amplified by 30 rounds of thermal cycling (1 min at 94°C, 1.5 min at 44°C for the first five cycles and at 55°C for the last 25 cycles, 1.5 min at 72°C) using the recombinant pHEN1 vector containing scFvs (accompanying paper) as template and backward primer 5′-CGCGGCCCAGCCGGCCATGGCC-GGTACCATGGAGGTCCAGCTCGA-3′ and forward primer 5′-CCGGAATTCCTATGCGGCCCCATTCAGATC-3′. The PCR products were cloned into pHEN1 vector [14]. The amber stop codon preceding the c-myc tag was then replaced by a codon for glutamic acid as follows. The scFv genes obtained above were PCR-amplified as above except that forward primer 5′-TTGTTCTGCGGCCGCAG-CATTttcGAGTTTG-3′ was used (the codon to incorporate glutamic acid is in lower case). To construct the *hsp70-anti-poxn* scFv fusion genes, the PCR products (a *Kpn*I-*Eco*RI fragment covering the scFv fragments and the coding region for c-myc tag) were placed behind the *hsp70* promoter in P-element transformation vector pKB255 [11].

The vector pKB255 carries the visible marker gene mini-*white* (*w*). W^- flies that are transformed with this vector will have pigmented eyes and can therefore be easily selected.

The truncated anti-*poxn* antisense was obtained by PCR (30 cycles, each 94°C for 1 min, 49°C for 1 min 20 s, and 72°C for 2 min) using recombinant pKS(+) vector containing the cDNA of *poxn* [11] as template. The primers used were backward primer 5′-CGGAATTC-CAGTCAGCAGAATGCC-3′ and forward primer 5′-CGGGG-TACCTTTGAATGGGGCATTTACG-3′. The anti-*poxn* antisense fragment (a *Kpn*I-*Eco*RI fragment) was placed behind the *hsp70* promoter in P-element transformation vector pKB255 [11]. This antisense fragment is complementary to the sequences encoding the entire Poxn [11], except the sequences coding for the paired domain and the following seven amino acids.

All anti-*poxn* scFv sequences and the anti-*poxn* antisense construct were verified by dideoxynucleotide sequencing [15] prior to cloning into the pKB255 vector.

2.3. Drosophila stocks and fly culture

Genetic symbols not otherwise explained are described in [16]. Balancer stocks were as follows. FM7C: In (1) FM7, y^{31d}, sc^8, w^9, sn^{X2}, v^{of}, g^4, B as balancer for the X chromosome; SM: In (2LR) D, Cy, dp^{lvI}, pr, cn^2 as balancer for the second chromosome; and TM3: In (3LR) TM3, y^+, ri, p^p, sep l(3)89Aa, bx^{34e}, Ser, Sb as balancer for the third chromosome. Each balancer chromosome carries at least one dominant marker that makes it possible to determine, by genetic segregation and chromosomal linkage analysis, the chromosome carrying the P-insertion. [16]. W^{1118} strain was used for injection and served as negative control in all experiments. Flies were maintained at 18–25°C on standard cornmeal, sucrose, dried yeast, and agar medium supplemented with Nipagin and seeded with a suspension of live yeast.

2.4. P-element-mediated germline transformation

Transformation was carried out essentially as described [17]. Early embryos (0–30 min at 22°C) from the w^{1118} strain (w^- strain) were injected with DNA solutions containing 500 μg/ml of recombinant pKB255 harboring the anti-*poxn* sequences and 100 μg/ml of the defective P-element helper plasmid pUChsΔ2-3wc (donated by D. Cribbs). Adults derived from injected embryos (G_0 adults) were back-crossed individually to flies from w^{1118} recipient strain, and the adult progeny (G_1 adults) that exhibited the w^+ phenotype were selected. Chromosomes carrying the P-insertions and the relative copy number of insertions were determined by genetic segregation and chromosomal linkage analysis, using dominantly marked balancer stocks. The insertions located on the X, second and third chromosomes were, respectively, maintained as FM7C-, SM-, and TM3-balanced stocks [16]. Transgenic lines are designated as follows. The first number or the first two letters describe(s) the α-*poxn* gene carried by the line: 2, 4, 5 and AS stand for α-Poxn2, α-Poxn4, α-Poxn5 scFvs and antisense, respectively. The number or the letter that follows the first number or the first two letters identifies the chromosome on which the recombinant P-element vector has been inserted. The last number is to discriminate between different independent lines carrying the same construct on the same chromosome. For example, line 5.3.6 contains α-Poxn5 scFv inserted on chromosome 3, and line AS.X.2 harbors the antisense gene inserted on the X chromosome.

2.5. Staging of embryos and induction of hsp70-anti-poxn expression

Embryos were staged using two different protocols. For screening of transgenic lines, staging was exclusively based on timing as follows. Egg laying was allowed for 1 h at 25°C, and the plates with the eggs (0–1 h old) were left at 25°C for additional hours until embryos reached the appropriate age. When accurate staging was desired, staging of the embryos was based on the completion of the ventral and cephalic furrows as described [11]. Heat shocks were administered for the specified time and at the indicated age described in Section 3, using a previously published protocol [11]. Non-heat-shocked and heat-shocked embryos were left at 22°C to develop at least until the time that PNS was differentiated. Late embryos and first-instar larvae were then prepared for microscopic observation.

2.6. Preparation of embryos and larvae for observation of external sensory structures

Late embryos and first-instar larvae were prepared for microscopic observation according to Dambly-Chaudière and Ghysen [5], and the sensory structures were inspected as in [11].

2.7. Immunostaining of Drosophila embryos

0–16 h old embryos were subjected to a heat shock of 30 min at 38°C as above and then allowed to recover at 25°C for 0, 5, 10, 15, 20 and 30 min before fixation. Fixation and staining of embryos were as described [18] using α-c-myc-tag 9E10 antibody as primary antibody and Vectastain ABC kit (Vector Laboratories).

3. Results

3.1. Construction of heat-inducible anti-poxn genes and their introduction into the fly genome

The anti-*poxn* scFvs used in this study have been described by Hassanzadeh et al. (accompanying paper). The α-Poxn2 and α-Poxn4 scFvs react with both denatured and native Poxn with half maximal inhibition values of 100 nM and 40 nM for native Poxn, respectively. The α-Poxn5 scFv also recognizes denatured Poxn but either does not recognize native Poxn, or recognizes it with high half maximal inhibition value.

In order to express the scFvs in *Drosophila* in fusion with c-myc tag, the amber stop codon preceding the c-myc tag was replaced by a codon for glutamic acid in each of the scFvs. As expected, this mutagenesis did not affect the specificities and half maximal inhibition values of α-Poxn scFv antibodies.

Table 1
Results of P-element-mediated germline transformation of *Drosophila* with recombinant vector pKB255

Plasmid	Embryos injected	Adults eclosed	Survival frequency (%)	Fertile adults	Frequency of fertile adults (%)	Trans-formed	Transformation frequency (%)
pKB255 (α-*poxn2* scFv)	352	207	59	163	79	12	7.4
pKB255 (α-*poxn4* scGv)	346	110	32	79	72	3	3.8
pKB255 (α-*poxn5* scFv)	349	222	64	180	80	24	13.3
pKB255 (antisense)	364	224	61.5	184	82	14	7.6

Survival frequency, frequency of fertile adults and transformation frequency are, respectively, defined as (adults eclosed/embryos injected)×100; (fertile adults/adults eclosed)×100; and (transgenic adults/fertile adults)×100.

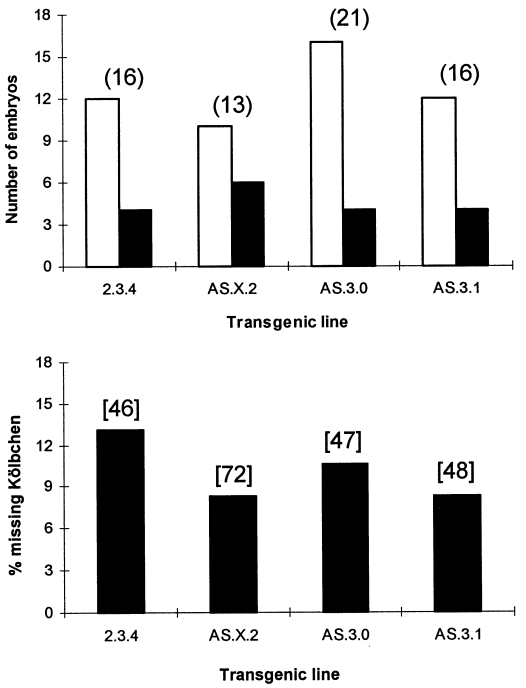

Fig. 1. *Poxn*$^-$ phenocopy resulting from the induction of the expression of α-Poxn2 scFv and antisense genes. Top: Expected (open bars) and observed (black bars) distributions of the number of embryos exhibiting *poxn*$^-$ phenotype following the induction of the expression of α-Poxn genes. Embryos exhibiting *poxn*$^-$ phenotype were scored by the absence of kölbchen. Embryos were obtained from crosses between flies heterozygous for the corresponding α-*poxn* construct. The expected numbers were calculated as the sum of the numbers of embryos that, based on Mendelian ratios, are expected to contain one or two copies of the α-*poxn* gene. Numbers in parentheses represent the total number of embryos analyzed. Bottom: The frequency of missing kölbchen in embryos exhibiting *poxn*$^-$ phenotype. The percentage of missing kölbchen was expressed as follows: (number of missing kölbchen divided by the total number of kölbchen examined in *poxn*$^-$ embryos)×100. Kölbchen which were not analyzable owing to imperfect mounting or clearing were disregarded in the calculation. They did not exceed 4.3% of the total number of kölbchen viewed within a group of embryos. Numbers in brackets represent the total number of kölbchen viewed.

Since these scFvs contain neither the hydrophobic secretory signal of the heavy and light immunoglobulin chains nor any other targeting signal, they are expressed as cytosolic proteins.

A truncated *poxn* DNA lacking the sequences coding for the paired domain and the following seven amino acids was selected for use as antisense. This is because the paired domain is largely conserved among a family of developmental control genes [12], raising the possibility that complete *poxn* DNA would interfere with the expression of paired domain genes other than *poxn*.

To allow the ubiquitous expression of anti-*poxn* genes, the antisense gene and the scFvs/c-myc constructs were placed under the control of the *Drosophila hsp70* promoter whose activity can be induced by heat treatment [19,20].

The anti-*poxn* elements fused to the *hsp70* promoter were introduced into the fly genome by P-element-mediated germline transformation. For each anti-*poxn* element, several independent, stably transformed lines were obtained (Table 1). The nomenclature used for transgenic lines is described in Section 2.

In all transgenic lines screened in this study, except in lines 2.X.0, 2.3.3, 2.3.4, 4.X.0, 5.3.0, AS.X.2 and AS.3.0, the *hsp70-anti-poxn* chromosome was homozygous lethal and could be maintained only over a balancer chromosome.

The results in Table 1 show that the transformation frequencies vary considerably for the different constructs. In general, the transformation frequency is in direct correlation with half maximal inhibition values of anti-Poxn antibodies. The antisense gene yielded a transformation frequency similar to that obtained with α-*poxn2* scFv gene.

3.2. Phenotypic screening of transgenic flies

During embryogenesis, *poxn* transcripts first appear about 5 h after fertilization at 25°C [12]. *Poxn* protein is first detected at the extended germ band stage (at about 5.5 h of development at 25°C) and remains detectable by immunolabelling until about 10.5 h [11].

To determine the phenotypic consequences of ectopic expression of anti-*poxn* genes, 4.5–5.5 h old embryos were heat-shocked at 38°C for 30 min. The embryos were then allowed to develop until late embryogenesis or the beginning of larval life. Late embryos and first-instar larvae were analyzed with respect to the locations and morphologic properties of their thoracic sensory organs. We concentrated on the thoracic segments because the kölbchen are much more conspicuous than their abdominal homologues, p6 and h3.

The screening of five independent transgenic lines containing α-Poxn2 scFv gene (lines 2.X.0; 2.2.0; 2.3.1; 2.3.3 and 2.3.4) identified one transformant, designated 2.3.4, in which the number of kölbchen was reduced after the heat shock. Similar results were obtained with three out of four lines harboring the antisense construct (Fig. 1). These three positive lines are designated AS.X.2, AS.3.0 and AS.3.1.

All transformants containing α-Poxn4 scFv gene (lines 4.X.0; 4.4.1 and 4.4.2), and transgenic lines 5.3.0, 5.3.6 and 5.3.8 transformed with α-Poxn5 scFv gene were screened as above. None of these lines showed a *poxn*$^-$ phenotype. The embryos were from parents heterozygous for the corresponding α-*poxn* insert. Based on Mendelian ratios, the results in Fig. 1 (top) suggest that only embryos that contain two copies of an α-*poxn* gene (about one fourth of the embryos) exhibit a detectable level of inhibition of *poxn*. The only exception is the line AS.X.2. Compared with other positive lines, a higher percentage of embryos from line AS.X.2 show the *poxn*$^-$ phenotype (Fig. 1, top).

In *Drosophila*, the amounts of the product of an X-linked gene are equal in males and females, despite the fact that the gene dosage in males is half of that in females. This phenomenon is called dosage compensation (reviewed in [21]).

When applied to w^{1118} embryos which served as negative control, the above heat shock regime affects neither the morphology nor the number of kölbchen. Non-heat-shocked embryos of lines 2.3.4, AS.X.2, AS.3.0 and AS.3.1 do not show any reduction in the number of kölbchen. These controls confirm that the *poxn*$^-$ phenocopy is indeed induced by the expression of α-Poxn2 scFv intrabody and antisense. With the exception of the hair h2 which was duplicated with a frequency of about 0.65% both in control w^{1118} and in transgenic embryos, none of the m-es organs analyzed was affected in heat-shocked embryos. This demonstrates that the inhibitory effects of α-Poxn2 scFv intrabody and antisense are specific.

The expression of *poxn* is maintained throughout the p-es lineage until about 10.5 h of development [11]. Using the

positive line 2.3.4, we also examined the effects of the late ectopic expression of α-Poxn2 scFv intrabody on the es organs. Induction of the expression of α-Poxn2 intrabody at 8 h 20 min to 9 h 20 min of development had no effect on the external morphology and number of kölbchen. Thus, it may be that *poxn* is only required at an early stage of development of kölbchen. Alternatively, the absence of any detectable effect of α-Poxn2 intrabody on *poxn* at later times can be explained by higher levels of *poxn* protein at these later stages.

In the experiments described above, the staging of the embryos was based exclusively on time. Although this method is rapid and relatively reliable, it does not stage embryos precisely because females may delay the laying of fertilized eggs. To control for this experimental variable, embryos from lines 2.3.4 and AS.3.1 were individually staged based on the completion of the ventral and cephalic furrows. Embryos were then heat-shocked at 38°C for 30 min at 4.5, 5, 5.5 or 6 h of development. Fig. 2 illustrates the results of these experiments. As shown in this figure, the *poxn*$^{-}$ phenotype is strongest when the inhibiting construct is expressed just before the appearance of *poxn*-expressing precursors.

3.3. Expression of anti-poxn intrabodies after heat shock

Immunolabelling of 2.3.4 embryos that show a *poxn*$^{-}$ phenotype did not detect the scFv protein after a 30 min recovery at 25°C from a 30 min heat shock at 38°C. This suggests that the amount of α-Poxn2 scFv protein in these embryos is below the detection limit of the immunolabelling technique. Since we observed no developmental defect associated with heat induction of α-Poxn2 expression in the other four lines transformed with the α-Poxn2 scFv gene (Section 3.2), we conclude that in these lines, the α-Poxn2 scFv protein is expressed at levels even lower than that in line 2.3.4.

All transformants containing the α-Poxn4 scFv gene and the transgenic lines 5.3.0, 5.3.6 and 5.3.8 harboring α-Poxn5 scFv gene were also screened by immunostaining as above. After 30 min recovery at 25°C from a 30 min heat shock at 38°C, ectopic accumulation of scFv protein could be detected in embryos from lines 5.3.0 and 5.3.6 (Fig. 3). These two lines express α-Poxn5 scFv intrabody throughout embryogenesis with a diffuse pattern typical of cytoplasmic proteins. The lack of *poxn*$^{-}$ phenotype in these lines (Section 3.2) is in line with the high half maximal inhibition value of this antibody for native Poxn or its reactivity with only denatured

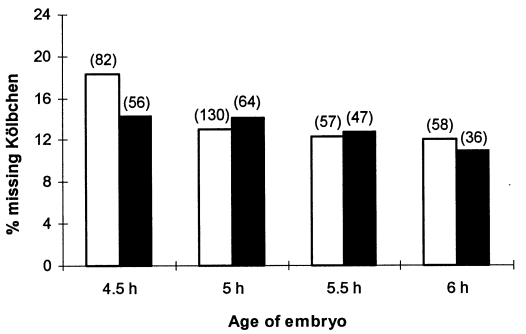

Fig. 2. Effects of the induction of the expression of α-Poxn2 scFv intrabody and antisense on *poxn* function in precisely staged embryos. The transgenic lines used are 2.3.4 (open bars) and AS.3.1 (black bars). The percentage of missing kölbchen is expressed as in legend for Fig. 1. Numbers in parentheses represent the total number of kölbchen viewed.

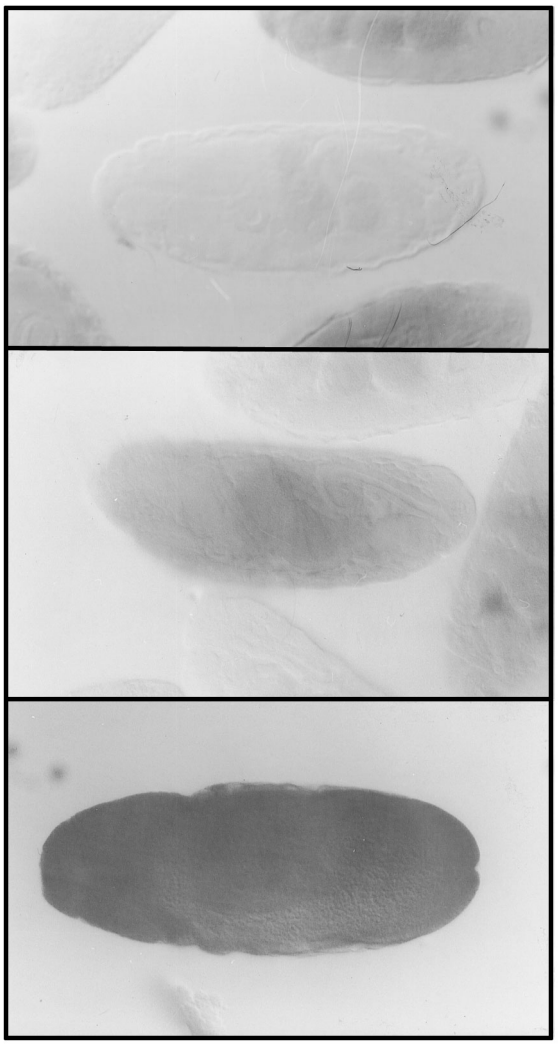

Fig. 3. Embryonic expression of α-Poxn5 scFv protein after a 30 min heat shock at 38°C followed by a 30 min recovery period at 25°C. Embryos were obtained from crosses between flies heterozygous for *hsp70*-α-Poxn5 scFv construct. It is therefore expected that 50% of embryos contain one copy, 25% contain two copies, and the rest contain no copy of a scFv gene. Among 67 embryos from line 5.3.0 subjected to immunolabelling, 55% appeared to be stained but weakly, compared with 27% of embryos which exhibited a staining of very strong intensity. The remaining 18% showed no staining. These figures are very close to those expected from the above-mentioned Mendelian ratios. An embryo representative of each of these fractions is shown. The entire embryos in upper panel, middle panel and bottom panel represent embryos which probably carry no copy, one copy and two copies of α-Poxn5 scFv gene, respectively. The embryos shown are from transgenic line 5.3.0. Similar results were obtained with the analysis of 81 immunostained embryos from transgenic line 5.3.6. Staining patterns of heat-shocked w^{1118} embryos (negative control) and non-heat-shocked embryos from lines 5.3.0 and 5.3.6 were the same as that of the entire embryo in the upper panel.

Poxn (see accompanying paper). The normal viability and phenotype of the embryos expressing the α-Poxn5 scFv intrabody shows that an intrabody per se is not toxic to a whole organism, extending the results obtained with cell culture assays which have shown that intrabodies are not toxic to cells.

At 30 min recovery at 25°C from a 30 min heat shock at 38°C, the levels of α-Poxn2 and α-Poxn4 scFv proteins are lower than those of α-Poxn5 scFv intrabody. This could be

either a result of the lower stabilities of α-Poxn2 and α-Poxn4 intrabodies or due to lower activities of the *hsp70* promoter in lines transformed with α-Poxn2 and α-Poxn4 scFv genes. To discriminate between these two possibilities, 2.3.4 embryos and embryos from lines transformed with α-*poxn4* gene were administered a heat shock of 30 min at 38°C, and then scFv accumulation at 25°C was monitored by immunodetection. No staining could be detected at 0, 5, 10, 15 and 20 min post heat shock. These results suggest that the low levels of α-Poxn2 and α-Poxn4 scFv proteins are, at least in part, attributable to the reduced activity of the *hsp70* promoter. The different strengths of the *hsp70* promoter in different transformants could reflect position effects of the sites of chromosomal insertion [22–24].

4. Discussion

Using three different α-Poxn scFv antibodies, we tested the possibility to express intrabodies in *Drosophila* and to specifically suppress the *Drosophila poxn* gene. In two independent lines, inducing the expression of one of these antibodies, α-Poxn5 scFv, during embryonic development, resulted in the cytoplasmic accumulation of the antibody throughout the embryo without causing any detectable toxic effects. However, this antibody did not inhibit *poxn*. This is probably due to the binding characteristics of this antibody, since either it does not recognize native Poxn or its half maximal inhibition value for native Poxn is very high (see accompanying paper).

Induction of the expression of α-Poxn2 scFv and anti-*poxn* antisense produced in embryos and larvae a phenocopy resembling the hypomorphic *poxn*$^{-}$ phenotype. The possibility that this mutant phenotype is due to insertional mutagenesis is ruled out by the fact that non-heat-shocked embryos of positive lines do not exhibit this defect. The inhibitory effect of these α-*poxn* genes is specific, since the induction of the expression of these genes did not affect the m-es organs whose developments are independent of *poxn*. The only non-specific developmental defect associated with heat shock of embryos harboring α-*poxn* genes was duplication of the hair h2 which was also observed with about the same frequency in heat-shocked embryos of recipient strain w^{1118} and is therefore entirely attributable to heat shock in this strain. The developmental defects caused by heat shock may result from the effects of heat shock on the transcription and/or translation of particular genes [25], and in some instances on RNA processing [26] and protein turnover [27].

These results demonstrate the feasibility of creating dominant, conditional hypomorphic mutations in *Drosophila* by cytoplasmic intrabodies. This warrants further studies aimed at evaluating intrabody technology in *Drosophila*, and also introduces intrabodies as a new addition to the repertoire of gene inactivation techniques in fly.

Assignment of the function to *poxn* during embryonic development has relied on the phenotypic analyses of embryos which either ectopically express *poxn* or are deleted for chromosomal regions that include *poxn*. The smallest chromosomal region analyzed in these studies is 17 kb, including the 8 kb of the *poxn* transcript [11]. The fact that this region is still more than twice as large as the *poxn* transcript and that breakpoints and deletions in *Drosophila* chromosomes can act over distances of kilobases [28,29] make it desirable to confirm by other methods that the phenotype observed in embryos deleted for the regions of chromosomes that contain *poxn* is indeed caused by the absence of *poxn*. The results of our experiments in which *poxn* transcript and *poxn* protein have been targeted, respectively, by an antisense and a Poxn-specific antibody further confirm that *poxn* is indeed a determinant of the p-es organs.

With the duration of heat shock used in this study (30 min), we obtained a hypomorphic *poxn*$^{-}$ phenotype by α-Poxn2 scFv. One reason for not obtaining an amorphic mutation may be that induction of the expression of this intrabody for 30 min does not yield the amount of antibody required to inhibit *poxn* completely. In *Drosophila*, maximum activities of *hsp* promoters are reached about 1 h after an increase of temperature (reviewed in [19]). We could not test the effects of the expression of α-*poxn* genes for longer periods of time, since heat shock regimes stronger than the one used in our experiments resulted in developmental defects in control w^{1118} embryos, complicating the assessment of the antisense and intrabodies effects.

The drastically reduced transformation frequency obtained with α-Poxn4 scFv, compared with that obtained with α-Poxn5 scFv whose expression does not inhibit *poxn*, indicates that even the basal level of the expression of α-Poxn4 scFv resulting from the leakiness of the *hsp70* promoter [30,31], used to drive the construct, is incompatible with the viability of embryos. Thus transgenic G1 embryos in which the recombinant P-element vector has been inserted in chromosomal sites favorable for efficient expression of α-Poxn4 scFv have probably been eliminated. This would explain why none of the transgenic lines harboring the α-Poxn4 scFv gene expressed the antibody at immunodetectable levels. The same holds for α-Poxn2 and the antisense construct. Compared with α-Poxn5 scFv, α-Poxn2 scFv and antisense genes yielded lower transformation frequencies.

The question raised here is: what make(s) these α-*poxn* genes lethal to organism? One possible explanation is that amorphic *poxn* mutations are lethal at one or another stage of the fly development, and therefore efficient inhibition of *poxn* by α-*poxn* genes with better inhibitory effects may account for these lethal effects. A systematic analysis of amorphic *poxn* mutations in both the PNS and CNS and their effects on the fly viability would allow to verify this possibility. It has already been demonstrated that an amorphic *poxn* mutation affecting the adult es organs is not lethal [32]. The second possibility is that the observed lethality may be related to non-specific toxic effects of α-*poxn* intrabodies and antisense. Two lines of evidence support the first possibility. First, there is a strong direct correlation between the inhibition efficiencies, estimated by inhibition ELISA, of the scFv antibodies and the extent to which they cause lethality. For instance, subtle differences in inhibition efficiencies of α-Poxn2 and α-Poxn4 scFvs appear to result in a significant difference in the effects of these antibodies on transformation frequency. It is worth noting that it has been reported that transfection of mammalian cell lines with a plasmid encoding an intrabody that potently inhibits an essential gene results in a dramatic reduction in the number of stable clones derived [33]. Second, in embryos from lines 5.3.0 and 5.3.6, ubiquitous accumulation of α-Poxn5 scFv which did not inhibit *poxn* was not lethal.

We do not exclude the possibility that the differences in the effects of these intrabodies on the viability of the flies may be

due to the interaction of these scFvs with different epitopes which can lead to differences in their inhibition efficiencies in vivo. Our experiments do not address this possibility.

As discussed above, when targeting a gene which is essential for the viability of *Drosophila*, the leakiness of *hsp* promoters may adversely affect the obtention of transgenic lines which express the intrabody at inhibitory levels.

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