TGFβ receptor saxophone non-autonomously regulates germline proliferation in a Smox/dSmad2-dependent manner in Drosophila testis

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Received for publication 1 December 2006; revised 23 April 2007; accepted 25 June 2007
Available online 3 July 2007

Abstract

Elucidating the regulatory mechanism of cell proliferation is central to the understanding of cancer development or organ size control. Drosophila spermatogenesis provides an excellent model to study cell proliferation since the germline cells mitotically amplify in a precise manner. However, the underlying molecular mechanism remains elusive. Germ cells derived from each gonialblast develop synchronously as one unit encapsulated by two somatic support cells (called cyst cells). Components of TGFβ pathway have previously been found to restrict germ cell proliferation via their functions in cyst cells. Here we report that saxophone (sax), a TGFβ type I receptor, is required in somatic cells to prevent the mitotically dividing spermatogonia from over-amplifying. Using various approaches, we demonstrate that Mad (Mothers against Dpp), a receptor-Smad usually associated with Sax-mediated TGFβ/BMP signaling, is dispensable in this process. Instead, Smox (Smad on X, Drosophila Smad2), the other receptor-Smad formerly characterized in TGFβ/activin signaling, is necessary for the precise mitotic divisions of spermatogonia. Furthermore, over-expressing Smox in cyst cells can partially rescue the proliferation phenotype induced by sax mutation. We propose that Smox acts downstream of Sax to prevent spermatogonial over-proliferation in Drosophila.

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Keywords: TGFβ signaling; saxophone; Smox; Germline; Proliferation; Drosophila

Introduction

Germline cells are one of the few cell types that maintain the ability to proliferate during adulthood. They undergo restrained mitotic divisions to transiently amplify themselves prior to entering meiosis and further differentiation. The precision of germline mitotic amplification provides an excellent model to study the mechanisms that control cell proliferation.

In Drosophila testis, the germ cells derived from one gonialblast develop in synchrony within the microenvironment created by two non-dividing somatic cyst cells (Fuller, 1998). The mitotic amplification that turns each gonialblast to 16 germ cells is tightly controlled. Germ cells at the mitotic phase are called spermatogonial cells. punt (ptu) and schnurri (shn), components of TGFβ signaling, have been found to function in the somatic cyst cells to restrict germ cell proliferation (Matunis et al., 1997). In other words, their effect is cell non-autonomous.

The versatility of TGFβ signaling in diverse tissues and at different developmental stages is conferred by the combinatorial complexity of the signaling components, especially the receptors and their downstream effectors (Feng and Derynck, 2005). The receptor for the TGFβ family ligands is a tetrameric complex composed of type I and type II subunits. The type II receptor Punt has been shown to function in the somatic cyst cells (Matunis et al., 1997). However, the identity of the type I receptor acting in this process remains to be identified.

Receptor Smad (R-Smad) is the mediator of the TGFβ pathway immediately downstream of the receptor (Derynck and Zhang, 2003). Drosophila R-Smad proteins are encoded by Mad (Mothers against dpp, or dSmad1) and Smox (Smad on X,
or dSmad2). In general, Mad is associated with TGFβ/BMP signaling and Smox with TGFβ/activin signaling (Brummel et al., 1999; Zheng et al., 2003, 2006). Shn, often found in the same complex with Mad, is involved in Dpp (a BMP-like ligand) signaling in different tissues and at different stages (Pyrowolakis et al., 2004). Being less selective, Put is implicated in both BMP- and activin-stimulated pathways (Marty et al., 2000; Zheng et al., 2006). In addition, Shivdasani and Ingham (2003) proposed that the control of spermatogonial proliferation by somatic cyst cells is mediated by Gbb, which is a BMP-like TGFβ ligand. Based on these previous findings in different tissues including testis, one would expect that Mad be required in somatic cyst cell to regulate spermatogonial proliferation. However, in a previous study Matunis et al. (1997) noted that clones mutant for Mad did not affect germ cell proliferation. This surprising result triggered us to thoroughly investigate Mad’s role in spermatogenesis.

In this report, we demonstrate that saxophone (sax), a type I receptor found in TGFβ/BMP signaling (Bangi and Wharton, 2006; Neul and Ferguson, 1998; Rawson et al., 2003; Shimmi et al., 2005), is required in somatic cells to restrict germline proliferation in Drosophila testis. Interestingly, using various approaches we confirmed that Mad is dispensable for the Sax-mediated signaling in this process. Instead, Smox is necessary for the precise mitotic divisions of spermatogonia, and Smox over-expression in cyst cells can greatly reduce germline over-proliferation in Smox mutant testes, or in testes carrying sax mutant clones. In the context of germline proliferation control, we discovered a yet unidentified connection between Sax, the receptor generally associated with BMP-like signaling, and Smox, the mediator ‘specific’ for activin signaling.

Materials and methods

Fly genetics

FRT42B sax/+; Cyo, babo16012/Cyo, Mad2−/Cyo, Mad−/Cyo, FRT ub/GFP tool stocks, UAS-FLP and Kr::GFP balancers were obtained from Bloomington Stock Center; Smox66/FM7 and UAS-Smox from Tzu-min Lee (Zheng et al., 2003); tkv/Cyo from Gary Struhl; FRT42B babo1/Cyo and FRT42B babo4/Cyo from Michael O’Connor; C587GAL4 from Ting Xie (Kawase et al., 2004); nosGal4VP16 from Ruth Lehmann; evg-lacZ, UAS-brk, FRT82B Med17/TM3 and Mad41/FRT40A/Cyo, act–>CD2–>GAL4-GFP from Richard Mann; and Mad2/FRT40A/Cyo from Dahua Chen. sax+ is a null allele (Glin114STOP of Sax-PA, personal communication with M. O’Connor) that has been characterized previously in various published studies (Flybase and references therein), and its lethality can be rescued by a sax transgene (Singer et al., 1997). All alleles of Mad have also been characterized and published previously (Flybase and references therein).

All crosses were set up at 25 °C except those crossed to Cyo. Over-proliferation phenotype was more frequent and consistent at 29 °C, and thus we did all experiments related to Smoxann at 29 °C. Smoxann, which carries a missense lethal mutation, appears to be temperature sensitive.

Generation of clones

To induce heat shock clones, newly emerged flies were kept in a 37 °C incubator for an hour and the testes were dissected after 5–6 days. Germline- or somatic cyst cell-specific clones were generated by expressing FLP with nosGAL4VP16 or C587GAL4 (Kawase et al., 2004), respectively.

Results and discussion

sax is required in the somatic cyst cells to control spermatogonial proliferation in Drosophila testis

The Drosophila genome contains three type I receptor genes, sax, tkv (thickveins) and babo (baboon). We examined the functions of all three in spermatogenesis by clonal analyses in which both germline and somatic clones were induced by heat shock. We found over-proliferation at a low frequency in testes carrying sax mutant clones (9 out of 105 testes carrying at least 1 germline GFP−/− clone) but not the other type I receptor tkv or babo mutant clones. The effect of sax mutation appeared cell non-autonomous because the germline sax−/− cells were apparently normal and the over-proliferating germ cells themselves were positive for the clone marker GFP, indicating they were not sax−/− (Figs. S1 and S2).

sax has been reported previously as being not required in germ cell proliferation (Matunis et al., 1997). Thus, to confirm our observations using mutant clonal analysis, we also examined the testes of homozygous sax mutation that survived to larval stage (L3) when spermatogenesis has already initiated and germ cells ranging from stem cells to pre-meiotic spermatocytes are clearly visible. BrdU labeling revealed that the germ cells were indeed undergoing excessive cell divisions (Fig. 1A). Germline-over-proliferation was also observed in the testes of homozygous saxP (a P-element allele; Xie and Spradling, 1998; Enhachiro Kawase, personal communication).

To determine in which cell type Sax exerts its functions, we induced mutant clones specifically in either germline cells or somatic cyst cells by expressing FLP specifically in the respective cell types (Fig. 1). We found that sax−/− germline cells are indistinguishable from their wild-type neighbors (Fig. S1), whereas germline over-proliferation was observed in the testes containing sax−/− somatic cyst cells induced by the cyst...
By cell size and DNA staining, these over-amplifying cells can be germline stem cells, gonialblast or spermatogonia. To determine the identity of the over-proliferating germ cells, we used a spermatogonial marker, branched fusome and a stem cell marker, *esg-lacZ* (Kiger et al., 2000). The presence of branched fusome and the absence of *esg-lacZ* staining in the over-proliferating cells confirmed their identity as spermatogonia.
proliferating cells demonstrate that these are spermatogonial cells instead of germline stem cells or gonialblast (Figs. 1B, C). Thus, we conclude that Sax is the type I receptor protein required in somatic cyst cell for the precise mitotic amplification of spermatogonial cells.

Although the sax mutation we used in this study is a molecular null allele (Gln114STOP, M. O’Conner, personal communication), we did not observe 100% penetrance in terms of spermatogonial over-proliferation. For example, homozygous L3 testes contained both over-dividing cells and normally developing germ cells at later stages (Fig. 1A). Using clonal analysis, we noticed that removal of sax function in cyst cells was not always associated with over-proliferation (Fig. S2 and data not shown). Since a low frequency of ‘tumor’ formation was also observed with babo mutant clones (tumor occurrence <2%, babo<sup>32</sup> is a loss of function allele; Table 1), we speculate that babo might play a minor role in this process along with sax. We also examined 2 other alleles of babo (babo<sup>164</sup> and babo<sup>16912</sup>) by inducing its mutant clones in cyst cells and found similarly weak effect on spermatogonial proliferation (n = ~200, tumor occurrence <2%). Thus, whether Sax functions with Babo or with a yet unidentified factor to transduce the signal in this process remains to be clarified.

**Bam is present in the over-proliferating spermatogonial cells induced by sax mutation in somatic cyst cells**

bam (bag of marbles) has been identified previously as the cell-autonomous regulator of spermatogonial proliferation. Removing bam in germ cells led to the over-proliferation of spermatogonia (Gonczy et al., 1997). However, we did not detect a lack of Bam expression in the over-proliferating spermatogonia induced by mutant sax clones in somatic cyst cells (Fig. 2). Therefore, the non-autonomous effect of Sax on germ cell amplification does not seem to work through regulating Bam expression. Similarly, Bam expression in spermatogonia was not affected when mutant clones of shn were induced in cyst cells (Matunis et al., 1997).

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**Table 1**

<table>
<thead>
<tr>
<th>genotype</th>
<th>Total testes</th>
<th>Testes containing over-proliferation</th>
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<tbody>
<tr>
<td>FRT42B sax&lt;sup&gt;4&lt;/sup&gt;/FRT42B ubiGFP; nosGAL4VP16:FLP</td>
<td>&gt;100</td>
<td>84&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>C587:FLP/Y;FRT42B sax&lt;sup&gt;2&lt;/sup&gt;/FRT42B ubiGFP</td>
<td>30</td>
<td>129</td>
</tr>
<tr>
<td>C587:FLP/Y;UAS-Smox FRT42B sax&lt;sup&gt;4&lt;/sup&gt;/FRT42B ubiGFP</td>
<td>166</td>
<td>2</td>
</tr>
<tr>
<td>C587:FLP/Y;tkv&lt;sup&gt;FRT40A&lt;/sup&gt;/ubiGFP FRT42B</td>
<td>151</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> This number also includes C587:FLP/Y;FRT42B sax<sup>2</sup>/FRT42B ubiGFP;UAS-X/+ testes, which display similar over-proliferation frequency to those without UAS-X and serve as the perfect control for C587:FLP/Y;UAS-Smox FRT42B sax<sup>4</sup>/FRT42B ubiGFP.

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**Fig. 2. Bam is present in the over-proliferating spermatogonial cells induced by sax mutation in somatic cyst cells**

**Fig. 2. Bam is present in the over-proliferating spermatogonial cells induced by sax mutation in somatic cyst cells.** All panels: only the apical portion of the adult testes is shown. Images of the same row were taken from the same sample doubly stained for Bam and DNA. Upper panel (WT): Bam protein are abundant in 2-, 4-, 8- and 16-cell spermatogonia around the apex (outlined by yellow dots). Lower panel (somatic sax<sup>-/-</sup>, genotype C587:FLP/Y;FRT42B sax<sup>4</sup>/FRT42B ubiGFP): the over-proliferating spermatogonial cells are revealed by bright DNA staining. The level of Bam in these cells is comparable to that in normal spermatogonia (outlined by yellow dots).
Mad is not required for the control of spermatogonial amplification

Mad has frequently been found downstream of Sax (Chen and Massague, 1999; Dorfman and Shilo, 2001) and in the same complex with Shn and Med upon Dpp activation (Chen and McKearin, 2003; Pyrowolakis et al., 2004), suggesting that Mad may play a similar role as Sax and Shn in spermatogenesis. However, Matunis et al. (1997) reported that heat-shock induced clones mutant for Mad did not affect germ cell proliferation. Since over-proliferation was induced at a low frequency in the testes carrying heat-shock clones of sax−/−, we suspected that the heat-induction of Mad clones was not efficient enough to display the phenotype.

To clarify the function of Mad in spermatogonial proliferation, we explored in different ways by knocking down or knocking out Mad activities in larval testes of different allelic transheterozygotes or in cyst cell clones, respectively (Tables 2 and 3). Interestingly, we did not find over-proliferation of spermatogonia in any of these testes, confirming the previous results. Similar to the germline clones of sax (Fig. S1) or shn (Matunis et al., 1997), germline clones of Mad mutation also appeared normal (Fig. S3). In addition, the immunostaining pattern of phosphorylated Mad, the activated form of Mad, is indistinguishable between wild-type and sax mutant testes (Fig. 3, and the negative control using Mad+/−, Fig. S4.). Thus, the effect of sax mutation does not appear to be mediated by Mad.

Over-expressing brinker (brk), a target gene repressed by Mad/Med/Shn complex, mimics the effect of Mad/Med/Shn disruption in the wing (Pyrowolakis et al., 2004). If Mad/Med/Shn complex is indeed involved in the signaling downstream of Sax in cyst cells, one would expect that Brk over-expression in cyst cells cause the same phenotype as sax or shn mutations. However, when we over-expressed Brk in the somatic cyst cells, spermatogonial over-proliferation was not observed (Table 2, n = 157 testes of hsFLP, UAS-brk/Y; act > CD2 > GAL4:GFP). This result is in accordance with our findings of Mad mutations in testis. Thus, multiple pieces of evidence obtained using various mutant alleles and different experimental approaches all support that Mad is not involved in the control of spermatogonial amplification.

Smox is the R-Smad required for proper spermatogonial proliferation

Mad and Smox are the only two receptor-Smad proteins encoded in the fly genome. We thus investigated if Smox functions in spermatogenesis. We used a mutant allele, Smox588, which carries a missense mutation and survives to L3 (Zheng et al., 2003). We examined a large number of Smox588/Y L3 testes and found over-proliferating spermatogonia in at least 1/3 of them (Fig. 3 and Table 3). Consistent with what we observed in sax mutant, Bam expression appears normal in the over-proliferating spermatogonial cells of Smox588/Y testes (Fig. 4).

To determine if Smox also functions in somatic support cells, we expressed wild-type Smox specifically in these cells in the Smox mutant background. Indeed, Smox expression in cyst cells reduced the occurrence of spermatogonial over-proliferation dramatically (Table 3). This rescuing experiment demonstrates that Smox acts in somatic cells to ensure proper amplification of spermatogonial cells.

To investigate if Smox mediates the downstream signaling of Sax activation, we tested whether expressing Smox could block the over-proliferation induced by sax mutation. Smox expression in cyst cells greatly reduced the frequency of spermatogonial over-proliferation elicited by sax mutation from ~36% to ~8% (Table 1). This suggests that Smox is

| Table 2 |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Mad is required in the control of spermatogonial amplification |
| | hsFLP; tubG4:GFP/Y;Mad512/FRT40A/ubiG80 FRT40A | hsFLP/Y;Mad512/FRT40A/ubiG80 FRT40A | C587:FLP/Y;Mad512/FRT40A/ubiG80 FRT40A | hsFLP; UAS-brk/Y;act > CD2 > GAL4:GFP/+ |
| Total testes | 151 * | 195 * | 136 | 157 * |
| Testes containing over-proliferation | 0 | 0 | 0 | 0 |

* Denotes the number of testes containing at least one GFP-positive cyst cell.

| Table 3 |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Smox, but not Mad, mutations led to spermatogonial over-proliferation |
| | Mad51-2/− | Mad51-6/− | Mad51-2/b1 | Mad51-2/12 | Mad51-2/− | sax4/4 | Smox588/Y | Smox588,C587/Y; UAS-Smox |
| Total testes | 22 | 80 | 37 | 43 | 44 | 37 | 58 | 128 | 140 |
| Testes containing over-proliferation | 0 | 0 | 0 | 0 | 0 | 0 | 53 | 48 | 8 |

Transheterozygous or homozygous mutants of Mad alleles could not survive beyond L3 (3rd instar larval stage), thus only L3 testes were examined. Different Mad alleles were balanced over GFP marked balancers. The severity of different Mad alleles is in the following order according to Flybase: 8-2 < f-2 < B1 < 12. All animals were raised at 25 °C except Smox588/Y and Smox588/C587/Y; UAS-Smox, which were raised at 29 °C. Over-proliferation phenotype in Smox588/Y was more frequent and consistent at 29 °C than at 25 °C.

* The control of this genotype is its sibling Smox588/C587/Y from the same cross. The percentage of control testes containing over-proliferation is similar to that of Smox588/Y.
likely a downstream effector of Sax in the proliferation control of spermatogonia.

Theoretically, Smox would not be functional in the absence of type I receptor kinase which activates its downstream Smads. How can we explain why over-expression of Smox rescued the over-proliferation phenotype induced by sax mutation? Since Smox has been demonstrated to be activated by Babo in promoting cellular growth and in neural development (Brummel et al., 1999; Zheng et al., 2003, 2006), it is possible that Babo can substitute this function in the absence of Sax in spermatogenesis. Indeed, our clonal analysis implicated that Babo is involved in regulating spermatogonial proliferation (Table 1).

Conclusions

Based on our current data and previous findings by other research groups, we propose a model on how the precise control of spermatogonial amplification is achieved (Fig. 5): As the two somatic cyst cells ‘escort’ the germ cells derived from each gonialblast through the spermatogenic program, they play a much more complicated role than just a physical shield. To prevent the mitotically dividing germ cells from over-amplifying, TGFβ type I receptor Sax (and Babo?), along with the type II receptor Punt and the transcription factor Shn, functions non-autonomously in the somatic support cells. However, Mad, the intracellular effector of BMP/TGFβ signaling is dispensable in this process. We provide evidence demonstrating that Smox is likely the effector downstream of Put/Sax to elicit a cascade of events in the cyst cells and then in the germ cells they surround. The communication and interaction between the two cell types makes the germ cells divide precisely four times. Such non-autonomous function does not work through maintaining the protein levels of Bam, the germline intrinsic factor required for proper cell divisions. However, we cannot exclude the possibility that Bam function is compromised post-translation-
ally. What exactly happens from the cell surface of the cyst cells to the mitotic machinery of spermatogonial cells is to be uncovered by efficient and targeted genetic screens.

Acknowledgments

We are deeply grateful to Dahua Chen, Ye-guang Chen, Tzu-min Lee, Ruth Lehmann, Richard Mann, Michael O’Conner and Ting Xie for sharing flies and reagents; to Bloomington Stock Center and Developmental Studies Hybridoma Bank at the University of Iowa for providing stocks and antibodies; to Joaquim Culi, Michael Crickmore and our lab members for their critical reading of the manuscript; and to our colleagues at IGDB for their support in many ways. This study was supported by the Bai-Ren Fund from the Chinese Academy of Sciences, and National Basic Research Program of China (#2007CB947503).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2007.06.019.

Fig. 4. Smox mutation results in spermatogonial over-proliferation. All panels contain images taken from the same L3 testis of Smox^{388}Y. (A) and (A') The apical end equivalent to that of adult testis is in focus and is indicated by a red arrowhead. Note the high expression of Bam in the normally amplifying spermatogonia (highlighted by yellow dots). Normal spermatogonia should be located only in a narrow region around the apex. (B and B') A tumor-like over-proliferation of Bam-abundant germ cells on the side of the testis is in focus (outlined by red dots). Their spermatogonial identity is revealed by their morphology, bright DNA-staining and Bam expression.

Fig. 5. Current model of germline proliferation control in Drosophila testis. This model is based on previous findings by other groups and our current study. Under normal conditions, a clone of germ cells (yellow dots) derived from each gonialblast develops in the environment encapsulated by two somatic support cells ("T" and "J" shapes in red). The number of germ cells reaches 16 after the precise mitotic amplification. Removing Mad function in either germline or somatic cyst cells shows no abnormality. In contrast, disruption of put, sax, Smox or shn in cyst cells ("T" shapes in light blue) results in germline over-proliferation. Bam protein appears abundant in the over-proliferating cells (yellow indicating Bam is on). Removing Bam in germline cells (empty circles) leads to over-proliferation cell-autonomously. How germline and somatic cyst cells interact to achieve the proliferation control remains to be elucidated.
References


