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Tissue, cell type and stage-specific ectopic gene expression and RNAi induction in the Drosophila testis

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Abbreviations: CySC, cyst stem cell; EGFP, enhanced green fluorescent protein; EST, expressed sequence tag; GSC, germline stem cell; HSF, heat shock factor; ORF, open reading frame; RNAi, RNA interference; tTA, tetracycline-responsive transcriptional activator; TRE, tTA response element; UTR, untranslated region; UAS, upstream activating sequence

The Drosophila testis has numerous advantages for the study of basic cellular processes, as production of sperm requires a highly orchestrated and complex combination of morphological changes and developmentally regulated transitions. Experimental genetics using *Drosophila melanogaster* has advanced dramatically with the advent of systems for ectopic expression of genetic elements in specific cells. However the genetic tools used in Drosophila research have rarely been generated with the testes in mind, and the utility of relatively few systems has been documented for this tissue. Here I will summarize ectopic expression systems that are known to work for the testis, and provide advice for selection of the most appropriate expression system in specific experimental situations.

Introduction

The *Drosophila melanogaster* testis is a wonderful organ for the study of many fundamental aspects of cell and developmental biology, as well as spermatogenesis per se. Production of sperm throughout adulthood is maintained via a carefully orchestrated cell division and differentiation program, starting with stem cells at the apical tip of the testis and resulting in release of motile sperm from the basal end. The process of spermatogenesis is amenable to cytological, molecular and biochemical analysis. The cell morphology of large cells with very well defined features facilitates phenotypic characterization of processes or defects by simple microscopy.

Drosophila has been established as one of the best model systems for fundamental biological research largely because of its advanced genetics.[1] Many mutations exist in stock centers, and mutagenesis screens are in place to try to realize the dream of having a loss of function mutant allele available for every gene.[2]

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However, there is of course much more to genetics than simple loss of function mutants. In Drosophila we can use ectopic expression systems to express genes of interest in specific cells. The ectopically expressed elements could encode the wild type protein, fluorescently tagged proteins, predicted gain of function alleles, homologous proteins from other species, hair-pin constructs for RNA interference (RNAi) and so on. One of the few disadvantages of spermatogenesis as a system to study basic processes is that you need the flies to survive until adulthood (or at least to late pupal stages). If the gene of interest is required for viability, or your ectopic construct induces lethality, then there will be no adults available for analysis. This problem can be avoided by using ectopic expression systems that can be controlled temporally and spatially to give conditional expression.

The Drosophila testis, despite its utility as a general system and clear relevance to reproduction, is relatively under-studied compared with other Drosophila tissues such as the ovary or the brain. These tissues are the subject of extensive research efforts, so many tools have been developed to allow ectopic expression of genetic elements of interest in specific cells. Fewer methods for ectopic gene expression have been developed for testes and those that have been developed have not been broadly publicised and hence are not widely used. Here I will discuss the systems that have been used for ectopic expression of genetic elements in testes. I will also outline some approaches that have not been successful. The intention is to provide a ready reference primer, to allow researchers who want to address the question "what does my gene do in testes" to formulate an experimental design for ectopic expression that allows them to answer the question.

A brief outline of *D. melanogaster* testis anatomy. The process of sperm production in *D. melanogaster* is comprehensively described elsewhere; and I will provide only a brief summary of salient features here, and refer the reader to these texts for the details.[3,4] Briefly, male germline stem cells (GSC) and somatic cyst stem cells (CySC) reside adjacent to a somatic niche structure, the hub. Interactions between these cells control stem cell numbers and cells displaced from the niche differentiate.[5] A fundamental differentiation unit, the cyst, is created when a spermatogonium is

encapsulated by two (now post-mitotic) cyst cells. Four spermatogonial divisions amplify the germline within the cyst. Dramatic cell growth occurs during the spermatocyte stage, before entry into the meiotic divisions. All 64 sister spermatids in a cyst differentiate together and in synchrony to result in a bundle of parallel, inherently asymmetric, elongated spermatids. After elongation the spermatids undergo an individualisation process and finally mature sperm coil at the base of the testis before passing into the seminal vesicle for storage until mating.

Developmental timing of gene expression. In terms of transcriptional activity in the male germline we can divide the spermatogenic process from GSC to sperm into three phases.[3] The GSCs and spermatogonia are similar in their gene expression profile. Some genes are activated as cells become spermatogonia, and presumably some are inactivated at this transition, but there is plasticity in the system as spermatogonia can, under appropriate conditions, de-differentiate and revert to stem cell identity.[6] Genes expressed in this first spermatogenic phase are, with perhaps very few exceptions, also expressed in at least one other cell type in the developing fly, for example the ovary.[7] The point of commitment to differentiation occurs as cells exit the mitotic cell cycle and become spermatocytes. This commitment is accompanied by a dramatic change in gene activity, with the expression of many testis-specific transcripts.[8] A large set of genes (approximately 10% of all predicted genes) are transcribed exclusively in the highly transcriptionally active primary spermatocytes.[7] Among more generally expressed genes there can also be a switch to expression of a testis-specific isoform, using either a novel transcription start site or a testis-specific splicing pattern.[9] Toward the end of the primary spermatocyte stage the cells shut down their transcriptional activity and then condense their chromatin for the meiotic divisions.[10] The final phase of spermatogenesis, post meiotic spermatid differentiation, occurs in cells that are transcriptionally much less active. There is limited post-meiotic transcription in *Drosophila melanogaster* spermatids, and a small set of genes are transcribed at a higher level in these cells than at earlier stages.[11,12] The cyst cells also undergo dramatic morphological changes and differentiate along with their germline cargo cells.[13] Obviously they have to expand to accommodate the cell growth of the germline, and then extend as the germline cells elongate. In early spermatid cysts, when asymmetry first becomes apparent in the germline one cyst cell is positioned adjacent to the clustering nuclei. This cell becomes the head cyst cell, remains relatively small and covers the spermatid heads, while the tail cyst cell elongates to ensheath the full length of the spermatid bundles.

Transcriptional control and translational controls work together to determine protein expression. With the fundamental knowledge of gene expression profiles described above we can begin to design expression systems to target ectopic gene expression to the desired cell type, at the appropriate stage of differentiation. However there is one confounding factor that is particularly pertinent to expression in the male germline—translational control. Because there is only limited transcription in spermatids the transcripts encoding proteins required for post-meiotic processes are almost all abundantly transcribed in spermatocytes.[14] The messages are stored in the cytoplasm in a translationally quiescent state until released for translation at the appropriate developmental time point. The details of this process are poorly understood, except that for the transcripts where the translational control element has been characterized it has been found in the 5' rather than 3' UTR. For example, the translational delay signal of the testis-specifically expressed gene, *djl,* resides in the 52 bp region between +43 and +95 of the *djl* transcript.[15] The translational control signals can be an irritation at an experimental design level, but also can become a component of the ectopic expression toolkit, as one can engineer a delay between transcription of the ectopic genetic element and its translation.

Established Methods for Ectopic Gene Expression

All the genetic constructs I will discuss are testis-specific or testis-biased implementations of standard *D. melanogaster* systems, and can be used to express any genetic construct of interest. These are very well described throughout the Drosophila literature and thus I will not revisit the generic setup of systems. The first decision to be made is whether to clone the ectopic fragment into basic promoter construct, and have it expressed under the control of the promoter and UTRs in the construct, or whether to chose a bipartite expression system such as Gal4-UAS and drive expression indirectly. Both methods have advantages and disadvantages. It should also be noted that transformation of *D. melanogaster* can now be achieved both via random integration and site-specific integration systems, and the user is advised to consider which is best for their specific application.[16]

Basic promoter constructs. In the simplest expression system, the gene of interest is cloned downstream of a promoter-enhancer module that directs expression in the desired pattern. This has the advantage of relatively simple genetics, as expression is achieved using a single transgene. Disadvantages include a lack of flexibility, since a new construct is needed for every expression pattern tested. Expression levels can also vary due to position effects is targeted insertion is not used. This expression pattern of transgenes driven by a specific promoter construct does not necessarily accurately mirror the expression pattern of the endogenous gene from which it was derived. Validation of expression patterns relies on experimental determination at both the RNA and protein level by using the promoter construct to drive a reporter such as LacZ or EGFP. The salient features of the basic promoter constructs described below are summarized in **Table 1**.

Beta-2-Tubulin based vectors. For testes the most well known basic construct, which is even colloquially known as the "testis vector," and can be used as an off-the-shelf solution is based on the *β-2-tubulin* (*betaTub85D*) regulatory sequences cloned in the P-element transformation vector pCaSpeR4.[17,18] The testis vector directs high expression of proteins exclusively in the male germline, beginning in late primary spermatocytes. It has been used in numerous studies, including the demonstration that human *BOULE* is capable of rescuing the meiotic defects caused by *boule* loss of function in Drosophila testes.[19] The testis vector contains about 2.1 kb of 5' genomic sequence, an EcoR1 cloning site, and 1.6 kb of downstream sequence. The ORF EcoRI site is

Table 1. Basic expression constructs suitable for testes (germline or soma)

Construct name	Promoter	5′ UTR	Intron	3′ UTR	Expressed in	Notes	Reference
Testis vector	β2tubulin (betaTub85D)	β2tubulin	No	β2tubulin	Mid-late primary spermatocytes		17
tv3	β2tubulin	β2tubulin	No	SV40	Mid-late primary spermatocytes	Fluorescent fusion protein variants available	22
pβ2tub	β2tubulin	β2tubulin	No	Hsp70	Mid-late primary spermatocytes		23
aly vector	aly	aly	aly 1st intron	aly	Early-mid primary spermatocytes	Fluorescent fusion protein variants available	Unpublished.
pCaSpeR-Hsp83	Hsp83	Hsp-83+user supplied	No	User supplied	Ubiquitous; perhaps enriched in germline.	Testis-specific transcription start site.	41
HSP83(5′-3′UTRs)	Hsp83	Hsp-83+cyt-c-d	No	cyt-c-d	Transcribed in primary spermatocytes, translated in spermatids	Might also be transcribed in other cells.	45
αtubulin-1 vector	alphaTub84B	alphaTub84B	alphaTub84B	SV40	Ubiquitous, weak	Fluorescent fusion protein variants available	38
Chip vector	Chip	Chip	Chip	User supplied	Ubiquitous	Promoter fragment length not described. Cloned into pCaSpeRN	39

inserted just 5' of the native AUG, thus any coding sequence should bring its own AUG. The insert should also contain a stop codon to avoid epitope tagging the ectopic protein with the last 12aa of *betaTub85D*. This construct was designed many years before the genome was sequenced, and a reanalysis indicates what else the genomic region contains. First, the 2.1 kb 5' region contains virtually all of the adjacent, ubiquitously expressed, transcription unit, *Task-7*, while the 3' region contains a testis-specific transcript (annotated as *HDC12658*,[20] but not yet included in the FlyBase annotation) encoding a short conserved protein of unknown function. Whether these have any bearing on?show=[ts]?>the expression of ectopic genes or have any other effects is not known. It is clear however that it is not necessary to have such extensive 5' and 3' genomic flanking regions to get testis-specific expression. Indeed testis-specific expression can be driven using a fragment from -53 to +156 (relative to the transcription start site).[21] A new testis vector, tv3, using a much shorter segment of *betaTub85D*, was developed.[22] A 0.6 kb fragment containing the promoter region and 5'UTR from *betaTub85D* was cloned into pUAST (replacing the UAS site), thus generating a construct from which the mRNA expressed will have *betaTub85D* 5'UTR- user derived ORF – SV40 3'UTR. A similar strategy was used to generate pβ2tub (also sometimes called pTMR), which has a 340 bp fragment of *betaTub85D* promoter and 5'UTR cloned into pGMT (in place of the Glass binding sites), and thus uses the 3' UTR from *hsp70*.[23] The level of expression of transgenes depends in part on the 3'UTR.[24] The *betaTub85D* 3'UTR gives high expression, but possibly with a slight translational delay. The expression from both newer vectors is also high, but pβ2tub might give slightly higher expression than tv3 (Julie Brill, pers. comm.).

Other alternatives for basic primary spermatocyte expression systems. The finding that an extremely short fragment of *betaTub85D* 5' sequence, spanning the transcription start site, is sufficient to direct testis-specific expression is the rule, rather than the exception, for testis-specifically expressed genes. The genomic sequences of several other genes have also been characterized, and short fragments are typically sufficient to drive transgene expression (reviewed in ref 7). All of these have been tested in a reporter construct system, and typically do not use the 3'UTR of the gene being studied. Many of the constructs tested also use an ectopic 5'UTR. For example, promoters tested in pCaSpeR4-AUG-βGal[25] have the promoter and 5'UTR from the gene of interest upstream of a fragment of *Adh* 5'UTR and first 30 codons of the *Adh* ORF, the *LacZ* coding region and the SV40 3'UTR and poly adenylation signal. As discussed above for *betaTub85D*, the 3'UTR can potentially alter the expression level of transgenes. More importantly for expression of ectopic proteins is the potential for translational control. BetaTub85D is a major component of the meiotic spindle, and thus of course is translated in primary spermatocytes. Most of the other well characterized testis-specific transcripts encode proteins that are not translated until spermatid elongation. Since control signals imposing the translational delay are typically found in the 5' UTRs, and deletion of the translational control signal leads to translation of the reporter protein in primary spermatocytes, it should be relatively trivial to generate a construct for ectopic expression using any of these characterized promoters. Inclusion, or exclusion, of the characterized translational control signals would give significant flexibility to experimental strategies in which the timing of protein expression is critical. Characterized promoters and UTRs with late translation suitable for this approach include *Mst87F*, *dj*, *djl*, *Mst36Fb*, *ocn* [15, 26–29]. Transcripts translated earlier in spermiogenesis include *Dpy-30L2* which is translated mid-elongation,[30] and *fzo*, which is translated at the completion of meiosis.[31]

BetaTub85D, and all the other genes mentioned so far are all transcribed in primary spermatocytes, but their expression peak is at late primary spermatocyte stages. To achieve earlier expression

of an ectopic element in early primary spermatocytes it is necessary to choose a promoter that is most active at this stage. Several good candidate early primary spermatocyte promoters exist, for example those based on the meiotic arrest genes.[7] The meiotic arrest genes encode regulators of testis-specific transcriptional activity, for example they are required for transcription of *betaTub85D* and the other testis-specific transcripts listed above. Their transcription initiates in early primary spermatocytes, and their mRNAs peak in early-mid spermatocyte stages.[32] We, in collaboration with Pier Paolo d'Avino and David Glover (pers. comm.), have used the *aly* 5' and 3' control regions cloned into pCaSpeR4 to create an ectopic expression system for early primary spermatocytes. Unlike the constructs discussed earlier, this includes an intron, contained within the *aly* 5'UTR. In general inclusion of introns is thought to aid the nuclear processing and export of transcripts and transcription, and thus is generally considered desirable in an ectopic expression system.[33] The construct was based on a 6.7kb genomic rescue fragment, and allows cloning of ectopic ORFs as a direct replacement of the *aly* ORF. A derivative of this vector contains the eGFP ORF, allowing expression of a C-terminally tagged eGFP fusion protein. Transgenes expressed from the *aly* vector recapitulate the normal *aly* expression pattern (HW-C unpublished). The drawback of this vector system is that the natural transcript level of *aly* is much lower than many of its transcriptional targets, so the ectopic protein is not expressed at particularly high levels.

Expression in post-meiotic cells. Until recently it was thought that virtually all transcription in the Drosophila germline was pre-meiotic, however it is now clear that spermatids are transcriptionally active.[11,12,34] We have generated constructs based on a set of post-meiotically transcribed genes that encode transcripts that are then localized to the growing ends of the spermatids, for example *schuy* and *c-cup*[35] (HW-C and Anuj Bhatt, unpublished data). We cloned *schuy* or *c-cup* genomic regions, with GFP inserted at the 3' end of the ORF into pCaSpeR4. We used approximately 1kb 5' of the transcription starts and 0.5kb 3' of the transcription stop sties. All the transgenic lines generated from these constructs recapitulated the native gene expression, indicting that the genomic regions selected carry all of the relevant translational control sequences. These constructs could be used to express ectopic proteins at the ends of elongating spermatids by simply replacing the *schuy* or *c-cup* ORFs with the gene of interest. The caveat here is that the ORFs might contain regulatory signals, which would be missing in the transgenes. In principle it should be possible to express a transgenic protein along the length of spermatids using these post-meiotic promoters and deleting the mRNA localization signal, however the mechanism of mRNA localization has not yet been determined, and it is not clear if translation is linked to the localization. For high level expression of ectopic proteins in spermatids it would probably be better to use a vector based on a gene expressed in primary spermatocytes, but not translated until spermiogenesis as discussed above.

Constructs for ubiquitous expression. There are a range of experimental situations where expression in the male germline is essential, and expression in the soma is either also desired or at least is not detrimental. On these occasions it could be appropriate to use a system that delivers ubiquitous expression. A large number of genes are expressed ubiquitously in the fly; however, some use different promoters to drive primary spermatocyte expression than they do to drive expression in other cells. At least three promoter construct systems are available that work well in all cells in the testes.

alphaTub84B (*alpha1-tubulin*) is ubiquitously and highly expressed, and apparently encodes a single transcript. The *alphaTub84B* promoter and 5'UTR (including the first intron) (-157/+696) has been cloned into pCaSpeR4, along with the SV40 3'UTR to generate a vector suitable for ubiquitous, low to moderate level expression of transgenes.[36,37] Derivatives allowing expression of fluorescently tagged fusion proteins (N-terminal mCherry or C-terminal EGFP) were generated by,[38] who were able to analyze fusion proteins expressed in primary spermatocytes.

CHIP is also ubiquitously expressed, at a lower endogenous level than *alphaTub84B*. A pCaSpeR-based vector containing the *CHIP* promoter and first intron has been used to drive expression of a *Su(Tpl)* transgene. This rescues the lethality of *Su(Tpl)* mutants, thus confirming its somatic expression.[39] It also rescues the sterility associated with some alleles of this locus, demonstrating its utility in expression of ectopic genes in the germline[39] (HW-C unpublished data). The expression level achieved using this system has not been determined.

Hsp83-based promoters are also useful for ubiquitous expression. *Hsp83* is endogenously ubiquitously expressed, and is mildly heat shock inducible in the soma.[40] *Hsp83* uses two different transcription start sites, as shown by ESTs and by the ModENCODE RNaseq data sets. The single annotated transcript *Hsp83-RA* corresponds to a form predominantly expressed in testes. The transcription start site used in embryos and other somatic tissues is about 150bp downstream of this. Precise details of the promoter construct used in the pCaSpeR-Hsp83 are not included in the paper describing it.[41] However the construct was based on the understanding of the transcription unit derived from studies in embryos.[42] The basic pCaSpeR-Hsp83 vector only contains the *Hsp83* "promoter," and not UTRs, so the 5' and 3' UTRs as well as the ORF need to be supplied by the user, for example as used to study myosin VI function in testes,[43] and the PI4-kinase *fwd*.[44] However, since testes (i.e., primary spermatocytes) use the upstream transcription start site, testis-expressed transcripts will have chimeric 5'UTRs. In principle, use of the vector without any additional 5'UTR sequences would probably bias expression toward the germline. A version of the *Hsp83* construct (HSP83(5'-3'UTRs)) includes the UTRs from the testis-specifically expressed gene *cyt-c-d*, in addition to the Hsp83 testis-specific UTR sequence, This confers the *cyt-c-d* translational controls on coding sequences cloned into this vector, and results in expression of proteins only in spermatids.[45]

Bipartite systems. In bipartite expression systems, the most commonly used being Gal4-UAS, a promoter-enhancer module that directs expression in the desired pattern is used to drive expression of an exogenous transcription factor (e.g., Gal4). The gene of interest is cloned, usually on a separate plasmid, downstream of the binding sites of the exogenous transcription factor

Table 2. Gal4 drivers lines suitable for testes (germline or soma)

Construct name	Promoter	5′ UTR	Intron	3′ UTR	Expressed in	Notes	Reference
nanos-Gal4-VP16	*nanos*	*nanos*	No	*nanos*	GSC, spermatogonia	Gal4-VP16 is FLAG-tagged.	53
bam-Gal4-VP16	*bam*	*bam*	No	*Hsp70*	Late spermatogonia, early spermatocytes		48
Hsp83-Gal4	*Hsp83*	*Hsp83-Hsp70*	No	*Hsp70*	Ubiquitous; perhaps enriched in germline		57
tub-Gal4	*αTub84B*	*αTub84B*	*αTub84B*	*Hsp70*	Ubiquitous		36
Act-5C	*Act5C*	*Hsp70*	*No*	*SV40*	Soma only	Strong	65
arm-Gal4	*arm*	*Hsp70*	*No*	*K10*	Soma only	Weak	67
C784		*enhancer trap*		*Hsp70*	Cyst cells		58
ptc-Gal4		*enhancer trap in ptc*		*Hsp70*	Cyst stem cells and cyst cells		60
Upd-Gal4	*Upd*	*Hsp70*	*No*	*SV40*	Hub		81
Elfless-Gal4	*Elfless*	*Hsp70*	*No*	*Hsp70*	Tail cyst cells		62

(UAS).[46] This introduces significant advantages in terms of giving spatial and temporal control to the ectopic expression system. It also introduces more complexity, including a time delay between onset of expression of the transcription factor gene and onset of the target gene expression, meaning the outcome of experimental strategies does not always match the prediction.[47] The genetics required to create strains with multiple inserts, makes this system more complex to use in practice. The salient features of the Gal4 lines described below are summarized in **Table 2**, and the expression patterns of key lines are depicted in **Figure 1**.

Using Gal4-UAS in testes—BetaTub85D-Gal4 doesn't work. As discussed earlier, *betaTub85D* is expressed specifically and very highly in primary spermatocytes, and testis vectors based on this are readily available. It is therefore obvious to use *betaTub85D* to establish the Gal4-UAS system in primary spermatocytes. Other obvious choices of Gal4 driver include Mst87F. It is not

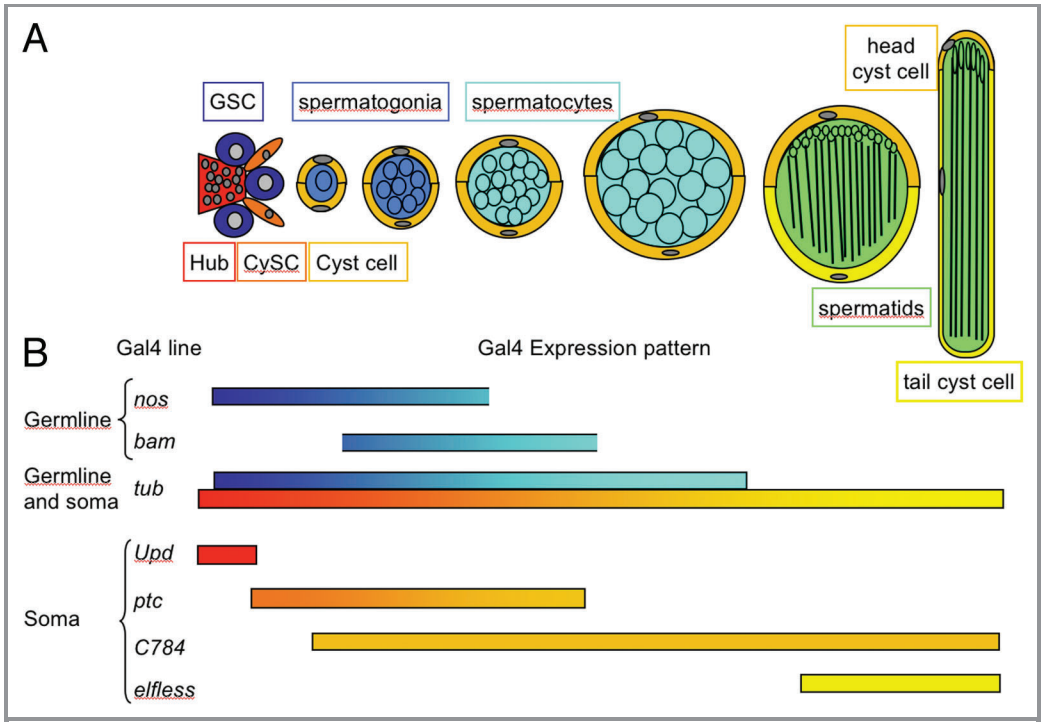

Figure 1. Schematic representation of expression patterns of Gal4 drivers in the testis. (A) Male germline stem cells (GSC, dark blue) and cyst stem cells (CySC, dark orange) contact the hub (red) a the testis tip. Spermatogonia (light blue) are encapsulated by cyst cells (light orange) and displaced from the hub. Spermatogonia differentiate into spermatocytes (cyan) which grow extensively before completing meiosis and becoming spermatids (green). Head and tail (yellow) cyst cells are distinguishable associated with elongating spermatids. (B) Different Gal4 driver lines can be used to drive downstream gene expression with spatial and temporal control. The bars indicate the developmental stage and cell type specificity of the expression of Gal4 in particular lines.

surprising that several labs have independently made *betaTub85D*-Gal4, *betaTub85D*-Gal4-VP16 or *Mst87F*-Gal4 (Steve DiNardo and William Mattox, pers. comm.), however these drivers apparently do not work. It is disappointing, but not surprising that the details of these ineffective constructs are not published. Thus exactly what has, and has not, been tried is not available for analysis. In practice the failure of these constructs to drive UAS-target gene expression frequently resulted in me being asked at conferences, in emails and in phone calls "why doesn't Gal4 work in testes?" Reassuringly the answer is "it does," see below. It is likely that the reason *betaTub85D-Gal4* and similar constructs don't work is related to the timing of expression of the Gal4 relative to the shut-down of transcription that occurs in maturing primary spermatocytes. There is probably insufficient time for Gal4 to accumulate to levels sufficient for driving target gene expression. Expressing Gal4 earlier in primary spermatocytes, or ubiquitously, should, and does, overcome this problem.

Using Gal4-UAS in testes – drivers that work. Personally, I was fortunate not to waste time and resources generating *betaTub85D*-Gal4, because I wanted to express exogenous genes in meiotic arrest testes and meiotic arrest testes do not express *betaTub85D*. Instead I tested drivers based on genes expressed independently of the meiotic arrest loci, and earlier in primary spermatocytes. The most successful has been *bam-Gal4-VP16.* This construct comprises the *bam* promoter (~900bp), *bam* 5'UTR, *Gal4:VP16* ORF and *Hsp70* 3'UTR cloned into pCaSpeR.[48] Gal4:VP16 fuses the transcriptional activation domain of VP16 to Gal4 and potentiates its activity. The native *bam* gene is expressed in spermatogonia, and accumulation of Bam protein is needed in male germline cells to activate the switch from spermatogonial divisions to primary spematocyte fate. While *bam*-Gal4-VP16 ; UAS-*bam*-EGFP rescues the sterility of *bam* flies, ectopic protein accumulation of transgenes driven with *bam*-Gal4-VP16 actually peak in spermatocytes.[48] Only one *bam*-Gal4-VP16 insertion line is published, and it is a homozygous viable insertion on the third chromosome. We have used it to drive expression of a variety of ectopic elements including tagged fusion proteins and RNAi hairpin constructs.[49,50] **Figure 1** shows the expression profile, both at the RNA level and protein level, of ectopic constructs driven by bam-Gal4-VP16. We find expression of targeted mRNAs and eGFP-tagged fusion proteins is typically first detected in 8-cell spermatogonial cysts, peaks in early-mid spermatocytes and declines as spermatocytes mature (**Fig. 2A–C**). The dynamics depends on the stability of the ectopic protein, and we have examples where the ectopic protein is detected in late-elongation stage spermatids (**Fig. 2D and E**), and others where the ectopic protein is exclusively detected in late spermatogonia and early primary spermatocytes (**Fig. 2F and G**). Two UAS vectors are routinely used for Drosophila, pUAST and pUASP. pUASP was designed because pUAST does not work in the female germline.[51] My experience is limited to transgenes cloned into either the original pUAST vector, or derivatives of it (e.g., pUAST-EGFP),[52] and the inability of this vector to promote gene expression in the female germline does not preclude its activity in the male germline.

To achieve germline-specific expression in males, starting from male germline stem cells, the best option is *nanos*-Gal4-VP16. This construct contains about 700bp of *nos* promoter, the *nos* 5'UTR, the FLAG epitope tag, Gal4-VP16, *nos* 3'UTRs and about 500 bp of genomic DNA 3' of the *nos* transcription unit. Expression of Gal4-VP16 mirrors expression of the endogenous gene in both male and female germlines.[53] *nanos*- Gal4-VP16 has also been shown to rescue the spermatocyte developmental defects caused by *pex2* loss of function.[54]

Other Gal4 drivers that also work in the germline. Several of the basic ubiquitously expressed promoter constructs are also available as Gal4 drivers, and have been demonstrated to work in the male germline. *alphaTub84B*-Gal4, usually referred to in the literature as tub-Gal4, is derived from the *alphaTub84B*-pCaSpeR4 vector and includes the *hsp70* 3'UTR.[36] This driver has been used in numerous studies, although very few of these involve analysis of testes. tub-Gal4 driven ubiquitous expression of *npc1*-EYFP was able to rescue both the lethality and male sterility of *npc1* mutants.[55] The sterility of *npc1* mutant males is due to cell autonomous defects in spermatid individualization, indicating the utility of this driver to activate expression of genes in the germline. For expression in the germline it appears that tub-Gal4 is less effective than *nanos*-Gal4-VP16, since the latter can drive UAS-*nclb* and rescue the fertility defect in homozygous null *nclb* mutant males while the former is unable to rescue fertility in *nclb* null animals, but can rescue a partial loss of function of this gene.[56]

Hsp83-Gal4, generated by cloning Gal4 into the *Hsp83* construct already described, has been used as a male-germline enriched driver. Originally it was used to show that spermatid individualisation depends on caspase activation, as male sterility, with a specific block in cystic bulge migration was caused when *hsp83*-Gal4 was used to drive expression of the caspase inhibitor p35.[57] The spermatid individualisation defect of *npc1* mutants could also be rescued using this construct to drive UAS-*npc1*-EYFP.[55]

Expression systems for somatic cells of the testis. The discussion so far has been limited mostly to expression systems in the male germline, however there are of course somatic cells that interact with the germline to ensure normal sperm production. It is obvious that the ubiquitous expression systems discussed above will drive ectopic gene expression in the somatic gonad, but what if your experiment requires expression exclusively in the soma, or in specific somatic cell types? Fortunately there are plenty of expression systems that fit the bill.

Gal4-UAS for specific testis somatic cell lineages. Several promoter constructs have been used to drive lineage-specific gene expression in the testis. The typical question being asked is a variation on "what cell type requires activity of my gene?." This question has been particularly important in understanding the regulation of cell division and cell fate determination in the stem cell niche, and thus the availability of drivers for specific cell types here (hub, cyst stem cells, cyst cells, male germline stem cells) is critical. Hrdlicka et al., 2002 examined the expression of 24 different enhancer-trap Gal4 lines in a variety of tissues, including the male genital tract.[58] They identified a set of lines that

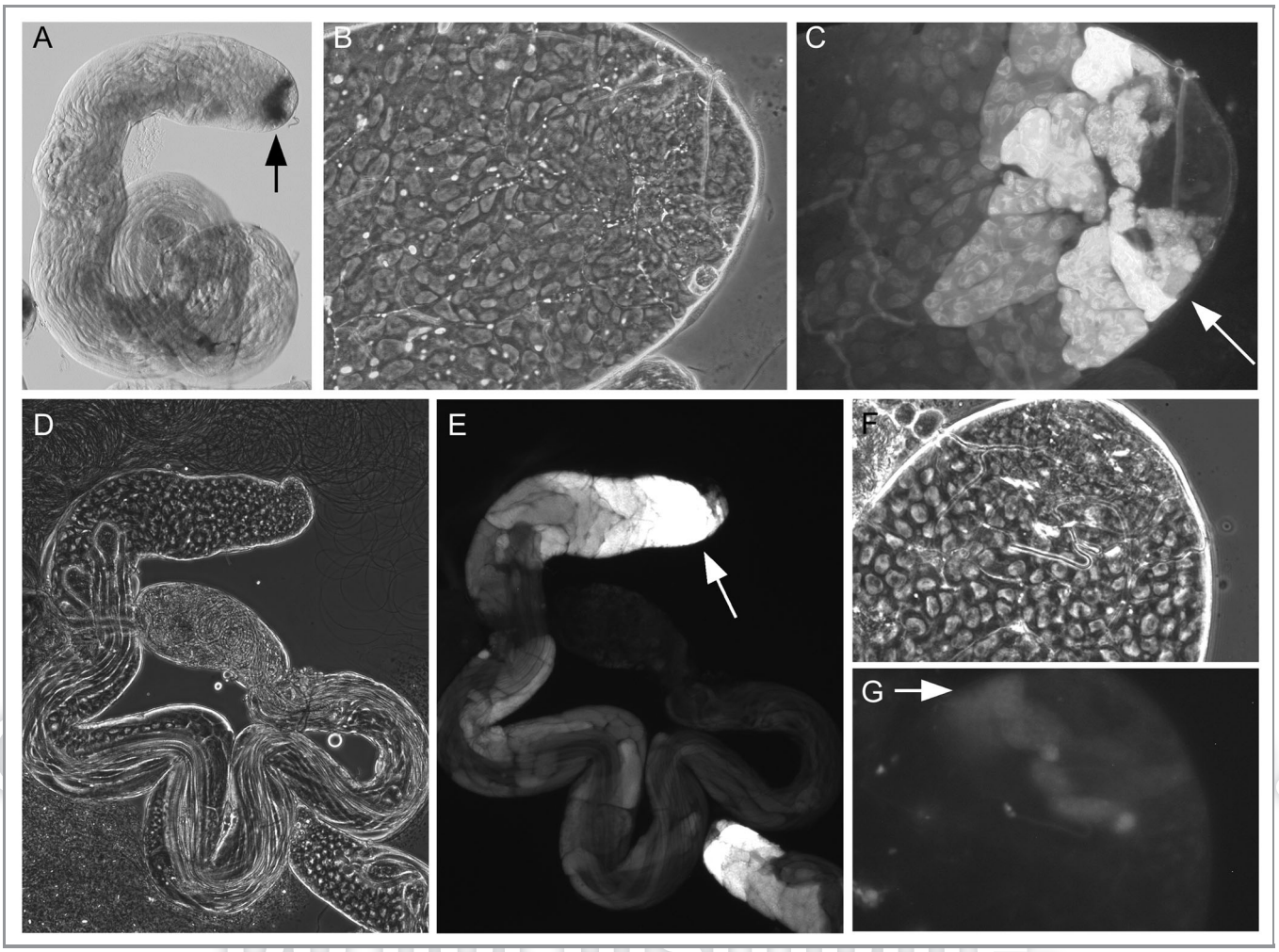

Figure 2. Expression of ectopic constructs using bam-Gal4-VP16. (A) RNA in situ hybridization revealing mRNA expression pattern for a pUAST-driven transgene (UAS-NLS-v5-TEV[79]). (B and C) Phase contrast and fluorescence images of a testis apical region revealing that when driven by bam-Gal4-VP16, EGFP-mip40 accumulates in early primary spermatocytes, and the expression declines as the spermatocytes mature. (D and E) Phase contrast and fluorescence images of bam-Gal4-VP16 driving UAS-EGFP-aequorin,[80] revealing perdurance into late spermatids of this stable protein. (F and G) Phase contrast and fluorescence images of a testis apical region showing that expression of an unstable protein (a defective form of Nxt1 tagged with EGFP) when driven by bam-Gal4-VP16 mirrors the driver expression pattern, i.e., the protein is detected only in late spermatogonia and early spermatocytes. Arrows in A, C, E, G indicate the testis region in which the driver is expressed.

could be used to drive ectopic gene expression, particularly in cyst cells (e.g., C784). Some of these lines reportedly also express in the male germline, although the authors note that the cell membrane localization of the EGFP used makes it hard to distinguish spermatocyte from cyst cell expression. To my knowledge these lines have not been used to direct germline expression in males. Cyst stem cell and early cyst cell expression can also be driven using a Gal4 enhancer trap insertion in *ptc* (P {GawB}ptc$^{559.1}$, usually referred to as *ptc*-Gal4).[59,60] Hub-specific expression in can be driven using *Upd*-Gal4.[61]

After formation of a spermatogonial cyst, the two cyst cells will remain with the daughters of that spermatogonium until the end of spermiogenesis, differentiating in concert with their encysted germline cells. The two cells initially appear equivalent, but after meiosis one will differentiate into a head cyst cell, associated with the end of the cyst containing the spermatid nuclei, while the tail cyst cell elongates extensively as spermatids elongate. Almost nothing is known about the mechanisms underlying this differential cell fate decision and their morphogenesis. There are relatively few markers for cyst cell differentiation, and thus few tools for cell type specific ectopic gene expression in late cyst cells. *Elfless*-GAL4 can be used to direct expression of transgenes specifically to the tail cyst cells.[62] *noa*, (encoding a fatty acid elongase) is expressed in a cyst stem cells at the testis tip, not expressed in early cyst cells, and then is re-activated in both cyst cells associated with post-meiotic germ cells. It is also expressed in the terminal epithelium cells at the base of the testis. While an expression vector has not been made for this gene, rescue constructs and a LacZ fusion reporter have been generated, and they recapitulate the native gene expression.[63] *noa* expression is not restricted to the testis, potentially complicating its use in experimental designs.

Ubiquitous somatic Gal4 drivers. Actin5C is ubiquitously expressed, including in the male germline, and the protein has been found as a component of the sperm proteome.[64] The *Act5C* promoter has been used to drive "ubiquitous" high level expression of Gal4.[65] However, at least in my lab, *Act5C*-Gal4 (we used the 17bFO1 insertion on chromosome 3) drives expression of transgenes exclusively in the somatic cells, and not in the male germline. This is surprising as the promoter fragment used was large (about 4.4 kb[66]), and there is no evidence that an alternative transcription start site for this gene is used in spermatocytes. In theory the should work equally well in soma and germline. In practice however, *Act5C*-Gal4 can be used to express transgenes at high levels specifically in somatic cells. Low to moderate levels of ubiquitous somatic expression can be achieved using the armadillo promoter, e.g., *arm*-Gal4.[67] Arm is a component of adherens junctions, and is expressed in all the somatic cells of the male genital tract, but is absent from the germline. Undoubtedly there are many more drivers that could be used to express ectopic genes in the somatic cells of the testis, however the male genital tract expression of most Gal4 lines that are available (over 600 stocks listed in Bloomington Drosophila stock center) has not been characterized.

To achieve short bursts of transgene transcription the classical system is to use a heat shock inducible promoter (*Hsp70*). This is activated on heat shock by heat shock factor (HSF). Heat shock induction of *Hsp70* was originally thought to not occur at all in testes,[68] however a more through analysis reveals that there is induction, but only in some cell types.[69] Specifically HSF is not detected in spermatocytes, and so heat shock induction is theoretically only possible in the somatic cells of the testes, the male germline stem cells and spermatogonia. Intriguingly, HSF is also detected in spermatid nuclei, raising the (untested to my knowledge) possibility that the heat shock system could be used to activate a burst of post-meiotic gene expression. Mason et al. (2002) used *Hsp70*-Gal4 driven expression of various *importin* α genes to look at potential functional redundancies, and found that the male sterility of *importin α 2* mutations can be rescued by *Hsp70*-Gal4 driven expression of the other importin α genes.[70] However, it is not clear if the fertility defect of *importin α 2* males is due to a germline or somatic requirement, and the cells in which the transgenes were actually expressed was not determined.

Expression of transgenes exclusively in the soma can help reveal gene function in the germline. We have taken advantage of the fact that well known "ubiquitous" Gal4 drivers are actually not expressed in the male germline to look at the germline function of ubiquitously expressed genes. Specifically, if a gene is required for viability then null mutations lead to lethality, and therefore there is no opportunity to examine testis-related functions. Expression of the gene of interest in the soma, using for example, *arm*-Gal4 > UAS-*gene*, allows rescue of the lethality, and thus generation of adult flies. Because *arm*-Gal4 does not express in the male germline it is then possible to examine the testes and deduce that any phenotype is due to loss of the gene activity specifically in the germline. This revealed a requirement for *Drosophila uri* in both male and female germline cells.[71] In testes the cell viability defect was partially, but not fully, restored by additional inclusion of *bam*-Gal4-VP16, pointing to a role for *uri* in the GSCs and/or spermatogonia as well as in spermatocytes.

Extra considerations for RNAi induction. All the systems described above are generally applicable, and not restricted to expression of a single type of ectopic element. The most common use of these systems to date has been expression of ectopic proteins, often with epitope tags or fluorescent fusion protein tags. The expression of such proteins can readily be monitored, and typically the expression data being sought is qualitative rather than quantitative. For example, the question might be "where does my protein localize?" or "does ectopic expression of my gene rescue the mutant phenotype?" In these cases it is rare that extremely high expression of the transgene is required. A more challenging use of the ectopic expression systems is to knock down gene expression using a hair-pin construct of the gene of interest to trigger RNAi. In this case high levels of the RNAi construct might be required to sufficiently deplete the endogenous mRNA and generate phenotypes. It can be particularly difficult to fully ablate the gene and protein expression and then analyze the null phenotype.

In my lab *bam*-Gal4-VP16 works for RNAi induction of UAS-hairpin constructs. We have tested RNAi constructs generated by the Vienna Drosophila RNAi Centre.[72] Their standard vector pMF3 has 10 copies of the UAS site in contrast to UAST, which only has 5 UAS sites. We found that we needed to push the expression system to the limits to get good knock-down of certain target genes. Specifically, to knock down *wuc* expression in spermatocytes we first tested the two independent transformants available, one on the second chromosome, the other on the third. The second chromosome insertion was homozygous lethal, while the insertion on the third was viable. Despite this we used the chromosome 2 insertion as it produced a higher level of knock-down. To knock down *wuc* (which is expressed specifically in spermatocytes) we had to generate male flies with the genotype *wucRNAi* / + ; *bam*-Gal4-VP16 / *bam*-Gal4-VP16.[49] Gal4 protein is inherently temperature sensitive and we noticed a dramatic variability in the phenotype depending on the temperature. When grown at 18°C the males were sterile, but the only defect in the testes was a lack of motile sperm. At 25°C there were defects in meiosis and spermatid elongation. At 29°C very few spermatocyte cysts progressed into the meiotic divisions, and those that did looked very abnormal. Finally at 30°C we generated what is probably the full loss of function phenotype, a complete failure of spermatocytes to progress into the meiotic divisions or spermatid differentiation (i.e., meiotic arrest). Control flies, expressing a hairpin RNA directed against *tin*, which is not expressed in testes, raised in parallel showed no defects in testis morphology. Flies raised at 30°C with just one transgene also retained their fertility; crosses were routinely set up using individuals that had been reared at 30°C. RNAi targeting of other genes using *bam*-Gal4-VP16, UAS-RNAi has yielded strong phenotypes even with both lines heterozygous and the flies cultured at 25°C (HW-C, unpublished data). In other labs the utility of *bam*-Gal4-VP16 and other drivers in spermatocyte RNAi has been variable. Di Cara et al. (2010) used a variety of drivers to express *Mst36F* RNAi transgenes, and were able to

induce a reduction in fertility but far from complete knockdown of the target gene expression.[73] A highly penetrant cytokinesis defect in male meiosis was induced using *bam*-Gal4VP16 and UAS-*anillin* RNAi,[38] however the same lab have not had such success with other UAS-RNAi lines (Julie Brill, pers. comm.). It is not clear if other labs have tested our finding that the degree of knockdown, and the severity of the resulting phenotype, can be highly dependent on the temperature at which the flies are cultured. In somatic cells it has been shown that co-expression of Dicer-2 can improve knock-down efficiency.[72] This has not been systematically tested in the testes, and it is possible that addition of a UAS-dicer-2 transgene will improve knock-down in spermatocytes.

In cases where the UAS-RNAi approach does not work it is possible to clone the RNAi hairpin construct directly into a testis vector. For this the *betaTub85D* constructs have proved very useful, for example, knock down of *fan* expression, although not complete, was achieved with a *fan* hairpin cloned into a *betaTub85D* expression vector.[74] In this case the RNAi-containing flies had reduced male fertility but were not sterile. More typically one would expect that constitutive expression of an RNAi construct in testes, of a gene required for fertility, would result in dominant male sterility. This is a complicating factor for stock maintenance as these insertions would either have to be balanced in trans to a dominant female sterile, or the transgenic females would have to be selected every generation to ensure continued propagation of the insertion.

Conclusions and Future Perspectives

Why some constructs work while others don't. Ectopic expression constructs in Drosophila originate via two independent routes. First, rational design is used, in which a construct is made based on the known endogenous expression pattern of a gene. The hope is that a region of genomic DNA from the region upstream of, and containing, the 5' end of the transcription unit will contain all of the control elements required to direct the local expression of the endogenous gene, and thus will work on transgenes. The second approach has been generation of an enhancer-trap transposable element, whose expression pattern is dictated by the genomic context in which it inserts.[46] Many Gal4 lines in use in Drosophila have come from screening of libraries of insertion lines of such elements. It is notable that all of the lines routinely in use for expression in the male germline have come from rational design strategies. The lines used for expression in the soma are derived from a mix of rational design and enhancer-trap screening. There are probably two reasons underlying this difference. First, relatively few (but notably not none) of the enhancer trap lines that have been generated have ever been tested for their expression in the male germline. Some might work, but we don't know about them. Second, the promoter architecture of genes expressed at high levels in spermatocytes is very different from that of most somatic cells. The expression patterns of spermatocyte-specifically expressed genes can frequently be recapitulated with fragments of just a few hundred base pairs.[7] Thus the proximal promoter region seems to be critical for spermatocyte expression and more distant enhancers are not typical. Enhancer trap screens depend on enhancers being able to work at a distance from a minimal promoter, and it is possible that this is not conducive to high expression levels in the male germline in general, and spermatocytes in particular. The rational design approach has been particularly successful probably because of the compactness of testis-specific promoters. Expression of transgenes in Drosophila male germline cells using a simple direct expression construct is relatively easy and reliable.

The use of Gal4-UAS in the male germline has been slightly trickier. A disappointing start for many was the finding that *betaTub85D*-Gal4 did not drive high expression of UAS-reporters. It is likely that this is merely a timing issue, the Gal4 from this construct probably does not accumulate sufficiently early to activate target genes before the general reduction in transcriptional activity that occurs as spermatocytes mature. This problem has been abrogated by using promoters to drive Gal4 earlier. The lines available are not yet ideal. The major outstanding item on the wish list is driver that expresses exclusively in spermatocytes (rather than also earlier), stays on throughout spermatocyte maturation (rather than declining), and expresses at high levels.

Also on the wish list would be the testing of alternative bipartite expression systems in the testes. Use of alternative systems could produce higher expression levels. More interestingly, with bipartite systems running in parallel it is possible to arrange situations with two (or more) transgenes being expressed in the same fly under different controls, e.g., one switching on before the other. Several systems have significant potential, and some have the advantage of conditional expression via inclusion of dietary supplements. The tet system uses the tetracycline-responsive transcriptional activator (tTA) that binds to the tTA response element (TRE). Tet-OFF is inactive in the presence of tetracycline, while tet-ON uses an alternative version of tTA that is active only in the presence of tetracycline. Both tet-ON and OFF have been established in Drosophila, although the basal expression from tet-ON has been a problem.[75,76] However neither version has been demonstrated in the Drosophila male germline. The recently developed Q-system, based on the regulatory genes of the *Neurospora qa* gene cluster uses the QF transcriptional activator binding to its target site to activate gene expression. Activity can be inhibited by expression of the QS repressor, and QS may be inhibited by addition of quinic acid to the culture medium.[77] In principle this system should work in the male germline, although this has not yet been tested.

The advent of inducible RNAi, in which expression of a hairpin RNA directed against the transcript of interest is induced in specific cells to specifically knock-down gene function has opened up the possibility of genetic analysis even in the absence of a mutant allele, and thus is a highly versatile tool.[72] Three large UAS-RNAi collections are now available, in Vienna (http://stockcenter.vdrc.at/control/main), Japan (http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp) and Harvard (http://www.flyrnai.org/TRiP-HOME.html) and transgenic flies containing UAS- long hairpin (Vienna, Kyoto, Harvard) or UAS- short hairpin

(Harvard) constructs are available for most predicted Drosophila genes. RNAi via expression of hairpin constructs does work in spermatocytes, but it is a challenge to get full knockdown of the target gene expression. This could be simply a downstream effect of not having sufficient expression, and thus could be solved by increasing Gal4 levels or activity (as the temperature increase does). Additionally, or alternatively, there could be inherent inefficiency in processing of long hairpin RNAs in these cells, as is the case in the female germline. If there is inherent inefficiency it should be possible to circumvent this using the short hairpin RNA design strategy that has been shown to work in the female.[78]

In conclusion, I have discussed the basic components of the molecular and genetic toolkit that have been shown to work for ectopic gene expression in Drosophila testes. Using these established methods it is possible to direct expression of user-defined genetic elements with high precision within this tissue. There are still methodological hurdles that preclude some aspects of experimental design, but in general it should be possible to design expression systems suitable for answering most questions about gene function within the testis. Expression can be directed ubiquitously, or to specific cell types within the gonad, and this expression can be achieved with either direct or bipartite expression systems.

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