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Expression of Gal4 alone alters DNA replication and causes cell death in ovarian follicle cells.

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Drosophila melanogaster is a powerful model organism for biological research in large part because of the many versatile genetic tools available to the fly geneticist. One of the most powerful tools is the Gal4-UAS system, which uses the yeast transcription factor Gal4 to drive expression of transgenic constructs within the developing organism (Brand and Perrimon 1993). This system has been expanded and modified to allow exquisite spatial and temporal control of expression (McGuire et al 2004). The importance of the Gal4-UAS system cannot be overstated. However, the utility of the system depends on the expression of Gal4 alone having no confounding effects on the cellular or developmental process being studied.

We have found that expression of Gal4 in ovarian follicle cells can result in disrupted developmental gene amplification, cell death, and altered egg chamber morphology. During oogenesis, the oocyte is surrounded by a layer of epithelial cells known as follicle cells which secrete proteins essential for chorion (eggshell) synthesis (see Calvi, 2006, for review). To support rapid eggshell synthesis, the DNA copy number of chorion and other genes are amplified by repeated rounds of DNA re-replication, a process known as developmental gene amplification. At Stage 10B of oogenesis, genomic DNA replication shuts down, and amplification begins at six discrete sites within the genome (Claycomb *et al.,* 2004). This amplification can be seen as nuclear foci of BrdU incorporation from stage 10B to 13 (Figure 1A, Calvi *et al.,* 1998). Surprisingly, we found that induction of an *Hsp70:Gal4* on the 3rd chromosome (Brand and Perrimon, 1993) alters BrdU

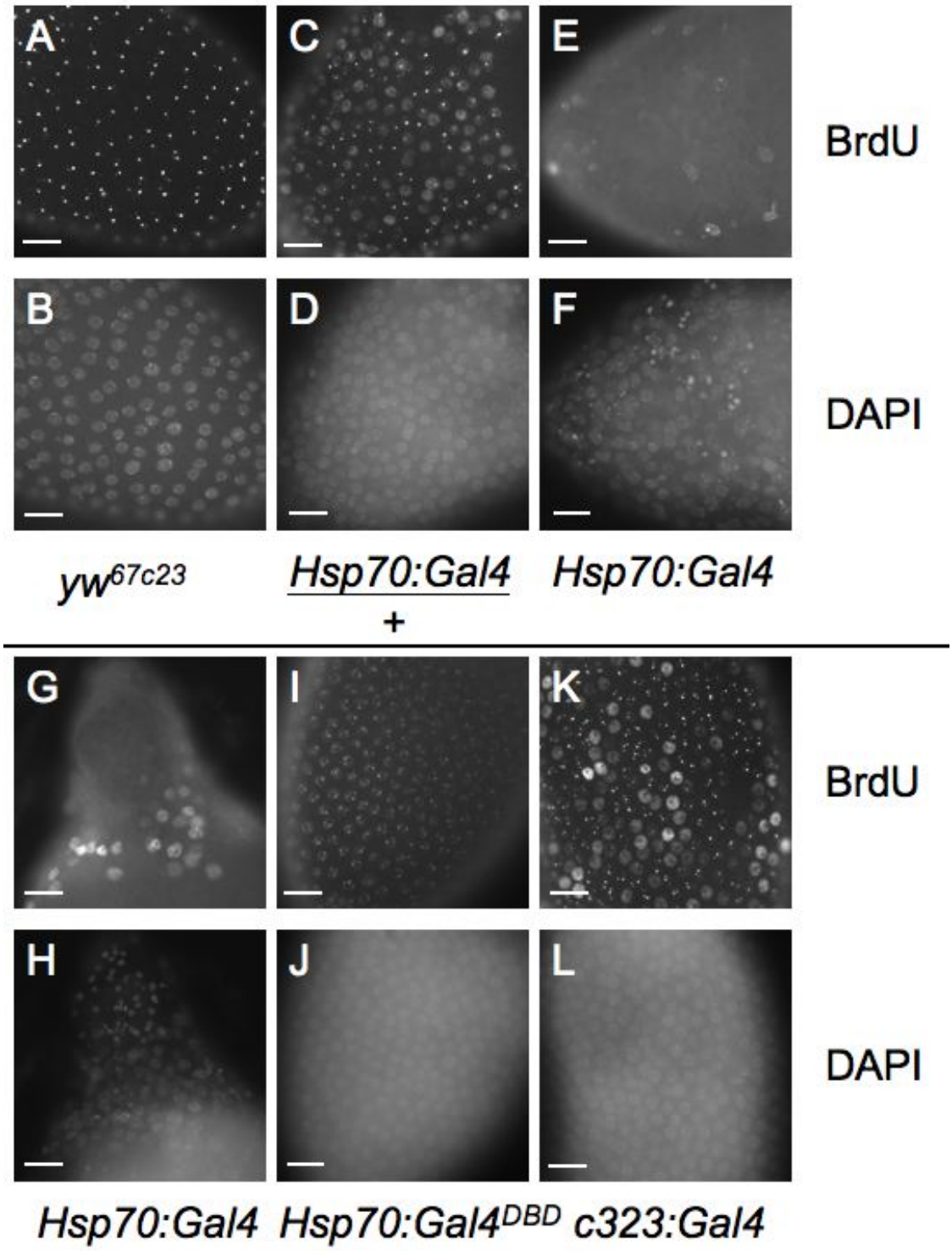

Figure 1. Adult females were heat-shocked for 30 minutes at 37°C, and allowed to recover for 14 hours at 23°C. Ovaries were dissected and labeled with BrdU (A, C, E, G, I, K) and DAPI (B, D, F, H, J, L) as described (Calvi *et al.,* 1998). Labeled ovaries were analyzed with a Leica DMRA2 fluorescent microscope. Genotypes analyzed include *yw*^{67c23} (A, B), *Hsp70:Gal4/+* (C, D), *Hsp70:Gal4* (E, F, G, H), *Hsp70:Gal4*^{DBD} (I, J), and *c323:Gal4* (K, L). Scale bars represent 10 □m in all pictures.

incorporation patterns 14 hours after heat shock induction. With one copy of *Hsp70:Gal4*, stage 10B egg chambers displayed a mixture of BrdU incorporation patterns in the follicle cells (Figure 1C). Approximately half of the cells displayed the normal BrdU focal pattern, while the remainder showed incorporation throughout the nucleus. With two copies of *Hsp70:Gal4,* BrdU incorporation is abolished in almost all follicle cells, and labeling with the fluorescent DNA dye DAPI revealed multiple pycnotic nuclei, suggesting that Gal4 expression causes cell death (Figure 1E-F). In addition, a subset of stage 9 egg chambers appeared misshapen, what we refer to as a "conehead" phenotype. In these egg chambers, the follicle cells collect at the posterior to form a tapered tip, rather than uniformly surrounding the oocyte as they normally would (Figure 1G-H). In addition, they appear to pile up on top of each other, instead of forming a normal organized epithelial layer. This suggests that Gal4 alters follicle cell migration and epithelial integrity during stage 9. These phenotypes occur with some delay and were not observed sooner than 12 hours after heat induction of Gal4 (data not shown).

The unusual BrdU incorporation and egg chamber morphology are not due to the insertion site of *Hsp70:Gal4*, as we observed that induction of an independent *Hsp70:Gal4* insertion on the 2nd chromosome (Brand and Perrimon, 1993) resulted in the same phenotypes (data not shown). Altered BrdU patterns and cell death were not observed after induction of a *Hsp70:Gal4*^{DBD}, which retains the DNA binding domain but lacks the transcriptional activation domain (Aggarwal and Calvi, 2004) (Figure 1I-J). Finally, we observed the same mosaic effect on BrdU incorporation 14 hours after heat shock of *c323:Gal4*, an enhancer trap line that expresses Gal4 in stage 8 and later follicle cells (Manseau *et al.,* 1997) (Figure 1K). While the most dramatic disruption of amplification occurred after heat shock, we have also observed altered BrdU incorporation patterns in some *c323:Gal4* egg chambers that were not treated with heat shock. Heat shock of control strains that do not contain a Gal4 transgene did not display these phenotypes, indicating that they are not due to heat stress alone (Figure 1A).

It remains unclear by what mechanism Gal4 affects DNA replication, cell viability, and egg chamber morphology. We are confident that these effects are not due to altered expression of genes at the insertion site as multiple Gal4 constructs on separate chromosomes display these same phenotypes. These effects also require the transcriptional activation domain, suggesting that altered transcription of endogenous *Drosophila* genes may be responsible. Consistent with this interpretation, a recent microarray study showed that over 1000 genes have significantly altered expression levels in larval salivary glands after induction of *Hsp70:Gal4* (Liu and Lehmann, 2008). Although these experiments were performed in different tissues, it suggests that the follicle cell transcriptome could also be drastically altered after Gal4 expression, which may lead to aberrant BrdU incorporation, cell death, and altered egg chamber morphology. Another possibility is that Gal4 may have toxic effects on the cell that are independent of its transcriptional activity.

The effect of Gal4 alone on fly cells is not unprecedented. It was previously shown that *GMR:Gal4*, which is expressed in larval eye imaginal discs, causes an increase in apoptosis and a rough eye phenotype (Kramer and Staveley, 2003). This further illustrates that Gal4 expression alone can have unforeseen effects in certain developmental contexts, which may confound the interpretation of experimental data. Our results emphasize that, although the Gal4:UAS system remains a powerful tool in *Drosophila* research, it should be used with appropriate caution and controls.

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Drosophila melanogaster mutant *tan*.

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Drosophila melanogaster gene *tan* was originally discovered in the early 20th century as a mutant strain lacking the dark pigment pattern of wild-type (wt) flies and, therefore, showing a light yellowish brown color (McEwen, 1918). Flies lacking Tan function also exhibited abnormalities in vision (Benzer, 1967; Inoue *et al.*, 1988; True *et al.*, 2005), and *tan* males displayed an abnormal courtship behavior (Cook, 1980; Tomkins *et al.*, 1982). tan^1 (t^1) and tan^3 (t^3) alleles were found as spontaneous mutations, t^3 mutant being apparently lighter than t^1 (Brehme, 1941). *tan* is the structural gene for N-β-alanyldopamine hydrolase (NBAD-hydrolase or Tan protein), the enzyme that generates dopamine (DA) from NBAD (Wright, 1987; True *et al.*, 2005). Tan is expressed as a precursor protein of 43.7 kDa. This precursor is cleaved into two subunits of 29.9 and 13.8 kDa that apparently conform together a heterodimeric active protein (Wagner *et al.*, 2007).

The enzyme that generates NBAD from DA, the opposite reaction to the one catalyzed by Tan, is the NBAD-synthase or Ebony protein (Wright, 1987; Pérez *et al*., 1997), which is codified by the gene *ebony*. Since both Tan and Ebony are involved in cuticle tanning, carcinine regulation, and NBAD metabolism in nervous tissue (Wright, 1987; Pérez *et al.*, 1997, 2004; Hovemann *et al.*, 1998; Borycz *et al.*, 2002; True *et al.*, 2005), it has been suggested that they function together in a system regulating the levels of dopamine during cuticle sclerotization and histamine in the visual metabolism (Borycz *et al.*, 2002; Pérez *et al.*, 2010).

During the last few years, several publications appeared regarding NBAD-synthase (Wappner *et al*., 1996a, b; Pérez *et al*., 1997, 2002, 2004, 2010; Hovemann *et al.*, 1998; Borycz *et al.*, 2002; Wittkopp *et al.*, 2002; Schachter *et al*., 2007), but very little is known about *tan* (True *et al.*, 2005; Wagner *et al.*, 2007). Thus, it was important to further characterize the NBAD-hydrolase in *D. melanogaster* wt and in mutants t^1 and t^3.

Methods

All *Drosophila melanogaster* wt (*CS*) and mutant (t^1; t^3; $ebony^4$ (e^4); $white^{1118}$ (w^{1118})) strains were from Bloomington Stock Center. *Ceratitis capitata* wt (*Argentina-17*) was from INTA-Argentina Stock Center. To study NBAD-hydrolase activity we developed a heterologous coupled assay for sequential synthesis and hydrolysis of NBAD. We performed both assays in 50 mM Tris/ClH buffer, pH 7.5 at 22ºC. For NBAD synthesis the reaction mix contained dopamine (0.1