Functional characterization of $dASCIZ$ in gene expression and development in $Drosophila melanogaster$

by

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DEDICATION

I dedicate this dissertation to my father and mother, who have always been my inspiration.
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ABSTRACT

A protein may perform various functions inside a cell through multiple signaling networks and help in fine-tuning cellular activities required to maintain complex physiology. A large number of proteins have been described that act as both stress response proteins as well as transcription factors in gene expression related to development. These functions may or may not be interlinked depending on the protein under consideration. For example, Sp1 was initially described to act as a transcription factor regulating the expression of a large number of essential genes. Later, it was also implicated in DNA damage response and in recent studies, has been shown to be a substrate for ATM-kinase-mediated phosphorylation with kinetics comparable to H2AX. Deletion of Sp1 sensitizes the cell to DNA damage induced by ionizing radiation. Like Sp1, Atmin (or ASCIZ) is another such protein with roles in both stress response and development. Atmin defines an NBS1 independent arm of ATM-kinase signaling under conditions of non-IR stress like hypotonic stress or oxidative stress. Studies from the knockout mouse work shows Atmin plays a crucial role \textit{in vivo} as a regulator of organ development during embryonic growth that is independent of its function in stress response. However, analysis of its role in post-embryonic development has not been possible as murine mutants studied so far, are embryonic lethals. In this study, we have characterized an EP- insertion line \textit{EP(3)3709} as \textit{dASCIZ} mutant and used it to investigate the roles of the protein during post-embryonic stages of 3rd larval instar and
metamorphosis (equivalent of adult development in sexually mature humans). *EP(3)3709* homozygotes survived till the pupal phase but did not eclose as adult flies. Mutant pupae displayed impaired metamorphosis with majority of the population showing little or no morphogenesis of adult head, wings, legs and abdomen. Expression of key ecdysone inducible genes including *Broad Complex* was significantly reduced in the mutant pupae compared to WT controls. Disruption of *dASCIZ* also affects DNA synthesis and cell division in mutant brains resulting in small size than the WT counterparts. The mutant larvae displayed a more transparent appearance than the WT controls suggesting a possible reduction in fat tissue. The increased transparency of the mutant larvae coupled with a complete failure to eclose suggest possible fat body malfunction and energy deficits. The lethality associated with the *EP(3)3709* homozygotes could be rescued by ectopically expressing the transgenic reporter protein dASCIZ-HA-mCitrine by the Act5C-Gal4 driver. This observation first established, *EP(3)3709* is a true mutant allele of *dASCIZ*. Second, it indicated, in absence of suitable antibodies capable of detecting endogenous dASCIZ, the behavior of the transgenic reporter protein dASCIZ-HA-mCitrine could be studied to predict the possible roles of the endogenous protein. Exploiting the presence of tags on the transgenic reporter protein (HA or Hemagglutinin in our case), the chromatin association property of dASCIZ-HA-mCitrine was analyzed by polytene chromosomes squash using anti-HA antibody. dASCIZ-HA-mCitrine localized broadly to the interbands regions of polytene chromosomes with an
extensive overlap of signal with active RNAPolII at multiple sites. In addition, dASCIZ-HA-mCitrine also showed a characteristic staining pattern in localization on polytene chromosomes before and after heat shock.

Analysis of roles of dASCIZ in metamorphosis and in the preceding stage of 3rd larval instar will give us a more holistic overview of its range of functions in a fruitfly model. Also our studies may help predict the involvement of its human counterpart in adult development.

On a broader scale, an understanding of the pathways involved in the two seemingly independent branches of Atmin function, DNA damage response and development, using different mutants alleles in different model organisms will provide a more detailed picture about the regulation of proteins with dual functions in gene expression and the DNA damage response.
CHAPTER 1. GENERAL INTRODUCTION

Background

ASCIZ (ATM substrate Chk2-interacting Zn$^{2+}$ finger) was originally identified as a stress-response protein involved in forming Rad51-containing foci in the event of Methylmethanesulfonate (MMS) induced DNA damage and not the canonical ionizing radiation (IR) induced dsDNA breaks. Subsequent studies showed ASCIZ interacted with the checkpoint kinase ATM (ataxia telangiectasia mutated) and based on this interaction, it was also called Atmin or ATM-kinase interactor protein. ASCIZ or Atmin (henceforth referred to as Atmin) was found to orchestrate an ATM-kinase dependent but NBS1 independent repair in response to agents that perturb the chromatin structure like chloroquine and osmotic stress. Subsequent studies also identified oxidative stress as a stimulus that required Atmin for full ATM activation. When cultured under atmospheric oxygen level (20%), Atmin$^{\Delta/\Delta}$ mouse embryonic fibroblasts (MEFs) proliferated normally during early passage (P2) but displayed a reduced rate of proliferation upon prolonged culture (P4). In addition, the Atmin$^{\Delta/\Delta}$ cells exhibited increased accumulation of DNA damage and higher levels of senescent marker β-galactosidase. Aging mice with CNS-specific deletion of Atmin (Atmin$^{\Delta N}$), displayed neurodegeneration in brain cortex, increased loss of dopaminergic neurons and impaired ATM-signaling.
Though Atmin and NBS1 were reported to regulate different stress specific ATM-kinase signaling pathways, both cooperate in maintaining genomic integrity. Spontaneous DNA damage was increased in \textit{nbs1Δ/Δ; atminΔ/Δ} double-mutant MEFs compared to the single mutants. Since ATM-interaction motifs of Atmin and NBS1 are quite similar to one another, it was argued that they interacted with the same region of ATM and competed with one another for ATM-kinase binding during stress. This argument was further strengthened by the observation that overexpression of Atmin led to reduced NBS1 mediated ATM kinase activation in IR stressed cells, a defect that was rescued upon NBS1 overexpression. This competition between cofactors is crucial for ATM-dependent signaling and loss of one cofactor was reported to increase the flux of ATM signaling through the other in the event of stress, causing serious biological implications. For example, in IR stressed cells, loss of Atmin increased NBS1 mediated ATM-signaling whereas in hypotonically stressed cells, loss of NBS1 increased Atmin mediated ATM-kinase signaling. Increased Atmin mediated ATM signaling is implicated in the cellular defects observed in the \textit{nbs1Δ/Δ} background, probably by altering activities of p53 and other substrates\textsuperscript{4}.

A report by Loizou \textit{et al} in 2011 described a role for Atmin in B cells. Not only was it required for the maturation of B cells but by using a mouse model lacking Atmin function specifically in the B cells (\textit{Atmin}\textsuperscript{ΔB/ΔB}), it was shown that, Atmin played a role in ATM-kinase mediated repair of breaks generated during...
peripheral V(D)J rearrangement and class-switch recombination (CSR). *Atmin*ΔB/ΔB mice developed B-cell lymphomas of heterogeneous nature thereby identifying a role for ATMIN in the maintenance of overall genomic integrity and tumor suppression in B cells⁵,⁶.

Later, Atmin was also found to be required for ATM-kinase signaling during replicative stress induced by treating cells with Aphidicolin. Aphidicolin is an inhibitor of replicative DNA polymerases α and δ and generates replicative stress by inhibiting the progression of the replisome⁷. Such a lesion activates both ATR (Ataxia Telengiectasia and Rad3 related Kinase) and ATM for resolution of fork defects, restart of replication and prevention of errors during mitotic segregation respectively. Details regarding the ATM mode of signaling were limiting. N Kanu *et al* in 2015 showed in a report that treatment of cells with aphidicolin led to the activation of ATM-kinase mediated signaling as evidenced by the formation of pATM (phosphor-ATM at Ser 1981) and 53BP1 (p53 binding protein 1) foci. The frequency of such foci formation was significantly reduced in *Atmin*-/- cells. Using HeLa cells containing a stably integrated *Lac O* (Lac operator) array and expressing a Cherry tagged Lac repressor (Cherry LacR), they generated a cellular set up with replicative stress. The Lac I protein bound to the *Lac O* repeats has been previously shown to impede replication and generate replicative stress⁸. In this set up, the stress site could be visualized as a single large focus by fluorescence from the Cherry tag. Rad18 ubiquitin ligase, WRNIP1 (WRN
interacting protein 1), pATM, and 53BP1 colocalized at the Cherry LacR site raising a possibility that they were participants of ATM-signaling. Indeed, ATM signaling was impaired in cells depleted of ATMIN or WRNIP1 or Rad18 (by using respective siRNAs) when stressed with aphidicolin but unaffected when stressed with IR. These observations showed that ATM kinase signaling follow a different route with a different protein set under replicative stress as compared to IR stress. Aphidicolin treated cells depleted of ATMIN also displayed a high frequency of γH2AX and UFBs (ultrafine bridges) as readout of unrepaired DNA damage and chromosomal abnormalities during mitosis. Such chromosomes when transferred to daughter cells will cause gross genomic defects and likely account for the increased frequency of tumor development observed in \textit{Atmin}^{AB/AB} knockout mice. Thus Atmin depletion impairs ATM signaling during replicative stress, compromises the fidelity of mitosis and affects genomic stability.

The human Atmin protein contains 823 amino acids with 4 ZNFs in the N terminus and an SQ/TQ cluster domain (SCD) with 17 potential ATM/ATR phosphorylation motifs in the C-terminus. Atmin as well as it SCD alone, can activate reporter gene expression in luciferase reporter and yeast-one-hybrid assays. Hence the SCD is also known as the transcription activation domain or TAD of Atmin. Using Chromatin immunoprecipitation (ChIP), ATMIN was found to bind to the promoter of \textit{Dynll1} (Dynein Light Chain 1) and regulate its expression in a ZNF dependent manner. DYNLL1 in turn interacted with Atmin.
by binding upto 10 out of its 11 TQT motifs in the TAD. Such interaction was important for repression of Atmin mediated $Dynll1$ expression in a negative feedback mechanism $^{11,12}$.

In connection with Atmin’s role in B-cell development, another report published by Jurado et al in 2012 showed absence of Atmin from B cells past the pro-B cell stage led to severe lymphopenia. This observation reinforced the idea that Atmin is required for B cell development. Rescue of this defect by ectopic expression of DYNLL1 in the Atmin null background and not by deletion of p53 or by complementation with a prearranged BCR (B cell receptor) indicated Atmin mediated transcriptional activation of $Dynll1$ and not DNA damage or defective V(D)J recombination was crucial for B cell development. At the pre-B cell stage, during B cell development, immature and transitional B cells expressing BCRs are eliminated by Bim (BH3-only protein) mediated apoptosis to prevent autoimmunity $^{13,14}$. Bim predominantly functions in the DNA damage independent cell death pathways and is a target of DYNLL1. DYNLL1 has been reported to attenuate the activity of Bim and keep the level of apoptosis under control $^{15}$. Such antagonism between DYNLL1 and Bim constitutes an important regulatory mechanism to maintain the delicate balance between immunodeficiency and autoimmunity. Interestingly, suppression of Bim activity in the $Atmin$ null background completely rescued B cell lymphopenia and exhibited splenomegaly like the $Bim$ only knockout mutants. Overall, these observations suggested that
reduced DYNLL1 expression in the Atmin null background from pro-B cell stage onwards, led to increased activity of the proapoptotic Bim accounting for reduced cell survival and lymphopenia.\textsuperscript{16}

The importance of Atmin mediated transcriptional activation of Dynll1 was further studied in developmental context when Atmin point mutant (mutation in the 3\textsuperscript{rd} zinc finger) mice \textit{Atmin}\textsuperscript{gpg6} displayed severe developmental defects including exencephaly and pulmonary defects.\textsuperscript{17} The pulmonary defects were characterized by small lung size and left isomerism. Previous reports have shown impaired cilia formation and function can affect pulmonary development.\textsuperscript{18,19} Indeed, nodal cilia, limb bud cilia and neural tube cilia displayed reduced length in the \textit{Atmin}\textsuperscript{gpg6} background compared to the WT controls. A significant reduction in the expression of Dynll1 as well as a small but statistically significant reduction in expression of some cilliogenic genes like \textit{Ift88}, \textit{Ift172}, \textit{Ift140} were recorded in the \textit{Atmin} mutant background.\textsuperscript{17} The severe down-regulation in the expression of Dynll1 is not surprising as the \textit{Atmin}\textsuperscript{gpg6} mutant contains a T to A transversion mutation causing a Cysteine to Serine substitution in the 3\textsuperscript{rd} ZNF of Atmin. Since Atmin was reported to regulate the expression of Dynll1 in a ZNF dependent manner, such mutation is expected to lead to a loss of transcriptional activation of Dynll1 by Atmin.\textsuperscript{11} A genetrap allele of Dynll1 (\textit{Dynll1}\textsuperscript{GT/GT}) was tested and found to manifest significant elements of the \textit{Atmin} \textit{gpg6} mutant phenotype like exencephaly, pulmonary isomerism and reduced nodal cilia length. This suggested
most of the phenotypes observed in the Atmin mutant background were triggered due to down-regulation of Dynll1 expression\textsuperscript{17}. In addition, Dynll1\textsuperscript{GT/GT} limb bud and neural tube cilia showed shortening and bulging, very reminiscent of the Dyn2ch1 (Heavy chain of cytoplasmic dynein 2 motor) mutant phenotype \textsuperscript{20,21}. Loss of Dynll1 likely had a negative impact on the function of cytoplasmic dynein 2 motor, which in turn affected retrograde IFT (intraflagellar transport) in the cilium causing the bulges. This argument was further supported by the observation that DYNLL1 interacted with WDR34, homologue of the Chlamydomonas cytoplasmic dynein 2 intermediate chain in co-immunoprecipitation studies\textsuperscript{17}. It is notable that DNA damage response was not compromised in the Atmin gpg\textsuperscript{6} mutant as was assessed by checking the immunostaining pattern and intensity of DNA damage marker 53 BP1\textsuperscript{22} between the wild-type and the mutant. This suggests that ATMIN’s contribution in embryonic development is independent of its function in DNA damage.

Atmin $\Delta/\Delta$ knockout mice generated by cre-lox mediated deletion of exon D was also reported to be embryonic lethal at E16.5 with absence of lungs, mid brain exencephaly and underdeveloped tracheas. Since ATM $\Delta/\Delta$ mice develop relatively normally compared to control, this observation, along with others, pointed to an ATM-independent branch of ATMIN functions\textsuperscript{10}.

In addition to lungs, ciliary dysfunction is also implicated in a number of kidney related disorders \textsuperscript{23,24}. Hence kidney development was probed in the Atmin gpg\textsuperscript{6}
mutant background\textsuperscript{25}. At E 13.5, mutant kidneys showed reduced size, disrupted branching morphogenesis leading to reduced number of epithelial bud tips and defective epithelial organization. While HH or Hedgehog signaling, ciliary structure and length, \textit{Ift88} expression as well as rates of growth and apoptosis in the mutant kidneys were comparable to the control, \textit{Dynll1} expression was severely reduced. In addition, mutant kidneys displayed disrupted actin cytoskeleton network akin to the mouse \textit{PCP} (Planar Cell Polarity) mutants implicating an aberrant PCP signaling\textsuperscript{26,27,28}. Previous studies have shown that the wnt-PCP signaling pathway is a critical regulator of cytoskeletal organization, which in turn is involved in shaping epithelial structures in both lungs and kidneys\textsuperscript{29,30}. Indeed, expression of \textit{β}-catenin of the canonical Wnt-PCP pathway and \textit{Daam2} and \textit{Vangl2} of non-canonical Wnt-PCP pathway were also found to be down regulated in the \textit{Atmin\textsuperscript{gpg6}} mutant background. Since ciliogenesis was not affected in the mutant kidneys, \textit{Atmin} probably regulated the development of kidney in a cilia independent manner. In light of the reduced \textit{Dynll1} expression, one hypothesis regarding \textit{Atmin} mediated regulation of kidney development involved dysregulation of the cytoplasmic functions of DYNLL1 in absence of \textit{Atmin}\textsuperscript{25}. This in turn was hypothesized to affect protein trafficking and orientation of MTOC (microtubule organizing center) and lead to PCP malfunctions, ultimately resulting in a defective kidney\textsuperscript{25,31}.

In 2014, \textit{CG14962} was identified to be the \textit{Drosophila} ortholog of \textit{Atmin} or ASCIZ (referred to as dASCIZ) encoding a protein of 388 amino acids with 4
ZNFs in the N-terminus and the characteristic SQ/TQ motifs of SCD with 6 TQTs in the C terminus. Like mammals, in fruitflies too, dASCIZ interacted with Cut-up or Ctp (Drosophila ortholog of human Dynll1) in a TQT dependent manner and regulated its expression through transcriptional activation of the ctp promoter. These observations suggested ASCIZ mediated regulation of expression of Dynll1 is evolutionarily conserved. Global knockdown of dASCIZ using the tubulin-GAL4 driver led to 1st instar lethality with no escapers. Tissue specific knockdown of dASCIZ in the posterior compartment (PC) of the wing discs by the en-GAL4 driver affected wing morphogenesis due to impaired mitosis and increased apoptosis. Interestingly, overexpression of ctp in the dASCIZ-RNAi background significantly rescued the defects of wing morphogenesis indicating dASCIZ functions upstream of ctp in the pathway for regulation of wing morphogenesis\textsuperscript{32}.

The null alleles of Atmin and the point mutants described in murine model are embryonic lethals\textsuperscript{10,17,25}. The global dASCIZ knockdown mutants described in the fly model are 1st instar lethals\textsuperscript{32}. This precludes any analysis of the physiological requirements of the protein during puberty or adulthood.

In our study, we have identified and characterized a P-element insertion line EP(3)3709 as dASCIZ mutants. Since the EP(3)3709 homozygotes never eclosed as adults and survived till the pupal stage displaying severely impaired metamorphosis, we used it as a platform to study dASCIZ’s contributions in metamorphosis.
Since antibodies detecting endogenous dASCIZ are still not available, we generated transgenic flies with dASCIZ-HA-mCitrine under UAS-Gal4 control. When driven by Actin5C-Gal4 driver, it rescued the lethality associated with the EP(3)3709 homozygotes. This suggested that EP(3)3709 is a true mutant allele of dASCIZ and the transgenic reporter protein could be used to study the behavior of endogenous protein. We used this system to study the chromatin association properties of dASCIZ under control and stressed conditions (heat shock) in both fixed and live preparations.

Overall, the goal of this study is to gain a more holistic understanding of the roles of Atmin by studying its Drosophila ortholog in a fruitfly model.

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Complex interactions between genes controlling trafficking in primary cilia.


**Research Questions and Significance**

**What is role of dASCIZ in post-embryonic development in flies?**

The mutants mentioned earlier, both in murine and fly models, died early in development before reaching adulthood, thereby precluding any analysis of its roles during adult development. In this study, we studied an EP element insertion line *EP(3)3709* as dASCIZ mutants that were not embryonic lethals but survived till the pupal stage making the analysis possible. The exact time at which lethality occurred was not precise and varied from very early metamorphosis with the formation of brown puparium with no detectable differentiation of tissue, to late metamorphosis with dead pharate adults. The majority of the mutant pupal
population displayed little or no morphogenesis of adult head, wings, legs and abdomen and displayed persistent salivary glands.

Previous studies have shown that ecdysone signaling orchestrated by the highly regulated expression of “early” genes and “late” genes, is crucial for proper metamorphosis to occur in flies. Aberrant expression of one or many ecdysone-controlled genes has been implicated in defective metamorphosis in multiple studies. To compare ecdysone response between age matched dASCIZ mutant and WT pupae, expression of a number of ecdysone regulated early genes was analyzed by RT-PCR at 0, 8 and 14 hrs APF (after puparium formation).

Since metamorphosis in insects is the transition from juvenile to sexually mature form, understanding the role of dASCIZ in ecdysone-mediated signaling pathway in fly metamorphosis may help us extrapolate the functions of its human counterpart in adult development processes involved in puberty.

The next question was if the dASCIZ-disruption mediated defective organ morphogenesis was restricted to the pupal stage or was it penetrant in other developmental stages. We looked into the preceding stage of 3rd instar larvae and investigated the morphogenesis of brains. Mutant brains were smaller in size than the WT controls. We then investigated if the small brains in the mutants was accompanied by reduced DNA synthesis (Bromodeoxyuridine or BrdU staining) and cell proliferation (Phospho-histone or PH3 staining) or increased cell death (Acridine Orange or AO staining). In addition, mutant wandering 3rd instar larvae appeared more transparent than the WT equivalents suggesting a possible
reduction in the amount of fat tissue. Reduction in fat tissue may be accompanied by energy deficits that may lead to a failure in eclosion and warrants investigation in the future.

**What are the subcellular distribution and chromatin association properties of dASCIZ?**

Analysis of subcellular distribution of a protein gives information about its nature of functions and constitutes an essential aspect of the overall understanding of the protein. The transgenic reporter protein dASCIZ-HA-mCitrine, when driven with suitable drivers, localized to the nucleus consistent with its human counterpart. Zinc-finger proteins have been shown to exhibit chromatin-binding properties. Information regarding the chromatin-association properties of Atmin (also a ZNF protein) *in vivo* is not available yet. Polytene chromosome squash is an elegant way of studying the association of a protein with polytene chromosomes (representative of interphase chromatin). Using this method, we found dASCIZ-HA-mCitrine was broadly associated with the interband regions of polytene chromosomes with an extensive overlap of signal with RNApolIIser5 at multiple sites. In doing so, dASCIZ-HA-mCitrine generated 2 types of staining patterns: i) Global staining (a relatively low intensity signal coming most of the sites of dASCIZ-HA-mCitrine occupancy on polytene chromosomes) ii) A discrete staining (Visibly increased signal from few selected sites of dASCIZ-HA-mCitrine’s occupancy on polytene chromosomes).
In addition, the transgenic fusion protein dASCIZ-HA-mCitrine also displayed a characteristic staining pattern on polytene chromosomes before and after heat-shock treatment.

**Generation of another mutant allele for a better understanding of dASCIZ:**

To generate another dASCIZ mutant allele, the EP element in *EP(3)3709/TM6b* flies was mobilized using the Δ2–3 transposase and screened for imprecise excision events that altered dASCIZ expression. One putative imprecise excision line was identified by its white-eye color. Preliminary PCR mapping suggested a small part of *EP3709* was retained while the major part was removed indicating an imprecise excision event. Cursory examination of the putative imprecise excision mutants (referred to as *IP3709*; IP for imprecise excision) revealed that *IP3709/IP3709* pupae displayed impaired metamorphosis and failed to eclose as adults like the *EP(3)3709/EP(3)3709*. Detailed examination of the *IP3709* line will add to the existing body of information regarding the contribution of dASCIZ in metamorphosis and may also reveal additional functions of the protein in gene expression, development and stress response. Comparison of multiple mutant alleles of the same gene is essential for a broader understanding of its functions.

**Thesis Organization**

**Chapter 1**

Summarizes the studies that have been done so far to understand the features and
functions of \textit{ATMIN/ASCIZ} and its ortholog in \textit{Drosophila}. It discusses the contributions of the protein in gene expression and development and highlights some of the areas in which information is limiting and/or lacking. Based on this, possible avenues of exploration regarding \textit{dASCIZ}’s chromatin association property and contribution in post-embryonic fly development have been briefly discussed.

\textbf{Chapter 2}

Literature review discusses fly development and the importance of ecdysone mediated signaling in it. It also briefly discusses the background of salivary glands and larval neuroblasts for they have been used to study chromatin association and cell proliferation respectively in our study.

\textbf{Chapter 3}

Details the functional characterization of \textit{dASCIZ} in gene expression and development in \textit{Drosophila melanogaster}.

\textbf{Chapter 4}

Discusses future directions of the project.
CHAPTER 2. LITERATURE REVIEW

Developmental transitions in the fruitfly *Drosophila melanogaster* and ecdysone response

The life cycle of the holometabolous insect *Drosophila melanogaster* is punctuated by 4 distinct developmental stages encompassing embryonic growth, juvenile growth, attainment of sexual maturation and adulthood. The 4 morphologically distinct stages are:

a. Embryo  
b. Larva (3 instar stages)  
c. Pupa  
d. Sexually mature, adult fly

At 25°C, the embryonic stage lasts for about 24 hours at the end of which, the eggs hatch into first instar larva. The first 2 larval instars also last 24 hours each. The last or the 3rd larval instar lasts for 48 hours approximately. The third instar larvae will pupariate if they reach a critical mass of about 0.3 mg. Under standard conditions, pupariation occurs at around 120 hrs after egg laying or AEL. Inside the cleidoic puparium, the remarkable process of metamorphosis occurs that transforms a larva into a sexually mature adult fly. The average life span of an adult fly is about 45-60 days at 25°C under optimal culture conditions. The progression through all these stages is hormonally regulated. Several classes of hormones are present in the fruitfly, each with its own distinct contribution to development. For example, the 20-hydroxyecdysone or 20E or β-ecdysone from the “ecdysteroid” group of hormones triggers the initiation of molting and metamorphosis and the “juvenile hormone or JH” controls the character of a molt.
These 2 hormones play a pivotal role in catalyzing fly development. In presence of JH, 20E can only catalyze the larva-to-larva transitions. At the end of the final instar, the JH titer drops and 20E can catalyze the onset of metamorphosis. Interestingly, the larval growth can be extended or terminated prematurely by external administration or deprivation of JH. Thus, the balance between the 2 hormones determines the developmental scenario. In addition, several peptide hormones (ETH or ecdysis triggering hormone, EH or the eclosion hormone) are essential for ecdysis of the old cuticle and final eclosion of a mature fly. In this section, we will focus on 20E ecdysone-mediated signaling in *Drosophila* development.

**Ecdysone signaling**

The major growth and molting hormone in *Drosophila melanogaster* is 20-hydroxyecdysone, commonly known as 20E. The chemically different α-ecdysone constitutes the minor ecdysteroids. Both the major and minor ecdysteroids are polar steroids and synthesized from dietary sterols as the fruitfly is incapable of synthesizing the steroid nucleus.

The primary ecdysteroidogenesis signal comes with the release of prothoraciotropic hormone or PTTH, a small neuropeptide secreted from 2 pairs of lateral neurosecretory cells that innervate the PG or prothoracic gland. PG is a component of the ring gland and the site of synthesis and release of ecdysone. PTTH, upon its release, stimulates the transcription of genes in PG committed to the synthesis of ecdysone. After synthesis, ecdysone is secreted into the
hemolymph and hydroxylated into the 20E form in the peripheral tissues (fat body, malphigian tubules, midgut, epidermis). Developmental programs such as formation of body plan during late embryogenesis, hatching into larva, molting at the respective larval instars, pupariation and metamorphosis into an adult fly, are characterized by 20E pulses\(^1\). A genetic signaling cascade that was first recognized as puffs in the giant salivary gland polytene chromosomes brings about the physiological changes in the animal. The cascade is a hierarchy of “early” genes and “late genes”, whose expressions are intricately linked to the 20E titer. Subsequent studies have uncovered the molecular details of the cascade and shown that an ecdysone receptor complex or ECR plays a vital role in transducing the 20E pulse to the target genes. The ECR is a heterodimer of Ultraspiracle (USP) and Ecdysone receptor (EcR). The EcR/USP\(^2\) (ecdysone receptor-ultraspiracle complex) binds 20E, associates with the ecdysone response elements in the promoters of target genes and controls their expression\(^3,4,5\).

The ecdysone-signaling mediated changes during different developmental transitions are distinct and elaborate. Such changes during metamorphosis will be briefly covered in this section. Metamorphosis in *Drosophila* is often categorized into a prepupal phase and a subsequent pupal phase. A pulse of 20E at the end of third larval instar triggers the puparium formation and catalyzes the prepupal development characterized by morphogenesis of adult legs and wings and apoptosis of larval midgut. A subsequent pulse at around 10 to 12 hours after puparium formation catalyzes the prepupal to pupal transition and is marked by
head eversion, onset of differentiation and histolysis of salivary glands. A final broad peak of 20E catalyzes the terminal stages of metamorphosis leading to the eclosion of the adult fly\textsuperscript{8}. The physiological changes are brought about by sequential activation of “early genes” and “late” genes. 20E in complex with its receptor directly activates the “early genes” (for example, \textit{E74}, \textit{BR-C} or \textit{Broad complex}). Their protein products, while turning off their own expression in a negative feedback mechanism, turn on the expression of 100 or so late genes, whose products effect the physiological changes of metamorphosis. Together they build, format and pattern an adult fly and constitute, what can be called, the core of ecdysone signaling. Therefore, any aberration in the expression of these genes will alter the signaling cascade, eventually impairing fruitfly metamorphosis\textsuperscript{9–12}. In this regard, some the ecdysone regulated early genes analyzed in this study, will be discussed briefly:

**\textit{E74}:** The early gene \textit{E74} is a member of the ets proto-oncogene family and encodes 2 related DNA binding proteins, E74A and E74B. The E74A and E74B proteins are composed of distinct N terminal regions and a common C terminal region containing an ETS DNA binding domain. Loss-of-function mutations studied in the \textit{E74} background are predominantly lethal during the prepupal and the pupal phases underlining the importance of the \textit{E74} gene products during metamorphosis. \textit{E74B} is crucial for both pupariation and pupation. The \textit{E74B} mutants die mostly as prepupae or early pupae. These mutants fail to form a normal puparium, suffer from impaired gas translocation and head eversion
indicating a defect in the functioning of the larval muscles. It is postulated that E74B prevents premature ecdysone-induced histolysis of abdominal larval muscles. The $E74A$ mutants, on the other hand, display some lethality in the prepupal period but die mostly as pharate adults. The $E74A$ mutants that die in the prepupal period are unable to evert their imaginal discs or undergo larval/prepupal apolysis. In addition, E74A is responsible for the full induction of multiple “late puffs” on salivary glands at the white prepuparium stage$^{13,14}$.

**Broad Complex: or BR-C:** The Broad Complex or BR-C is an early ecdysone response gene that plays a pivotal role in insect metamorphosis. BR-C is located at a cytological position of 2B5, near the tip of the X chromosome. It encodes a family of C$_2$H$_2$ zinc-finger proteins (Z1, Z2, Z3 and Z4). The zinc fingers share a common N terminus or the BR-C core and variant zinc-finger domains in the C terminus. Alternative splicing links the core with one of the 4 zinc-finger domains resulting in 4 classes of zinc-finger proteins. The BR-C locus contains at least 3 lethal complementing classes of alleles (Broad or “Br”, reduced bristle number on palpus or “rbc”, “2bc”), one non-complementing, non-pupariating class the “npr” and another partially non-complementing class, the “2Bab”. In general, the BR-C mutants display severely affected metamorphosis. The stage and tissue specificity of the impairment depends on the class of mutant being studied. Briefly, the $br+$ function is essential for tanning and sclerotization of larval cuticle as well as for appendage-eversion and elongation from imaginal discs. The $Rbp+$ function regulates muscle and bristle development. The $2Bc+$ function is crucial for the
fusion of discs to form adult epidermis. While the histolysis of larval tissues and
gut morphogenesis require both rbp+ and 2Bc+ functions, reorganization of the
CNS requires all 3 functions\textsuperscript{15}.

\textbf{\(\beta FTZ-F1\):} is an orphan nuclear receptor and serves as a competence factor in the
mid-prepupal phase of development. FTZ-F1 codes for 2 isoforms: \(\alpha\) FTZ-F1 and
\(\beta\)FTZ-F1. These proteins are characterized by a common C-terminal domain
containing the DNA binding and dimerization/ligand-binding regions and a variant
N terminal region. The \(\alpha\)FTZ-F1 is supplied as a maternal product and functions
as a co-factor of FTZ during segmentation of the growing embryo. In addition to
late embryonic stage, \(\beta\)FTZ-F1 is expressed in mid-prepupae under low 20E titer.
Its function in the mid-prepupal phase is to make the insect competent to respond
to the pulse of 20E at 10 to 12 hrs APF. \(\beta FTZ-F1\) mutants pupariate normally and
progress through prepupal development but suffer from severely affected
prepupal-to-pupal transition. The \(\beta FTZ-F1\) mutants studied, were characterized by
defective head eversion, leg elongation and persistent salivary glands, among
others. These events are hallmarks of the prepupal-to-pupal transition. In addition,
expression of early ecdysone genes, specifically \textit{E74}, \textit{E75A} and \textit{E93} but not \textit{EcR}
and \textit{Usp} was submaximal in the mutants at 12 and 14 hrs APF. Thus disruption of
\(\beta FTZ-F1\) interferes with 20E regulated gene expression during the prepupal-to-
pupal transition. \(\beta\)FTZ-F1 binds to many ecdysone-regulated puffs on salivary
chromosomes of late prepupae, including sites bound by the ECR-USP
heterodimer. It is hypothesized that that it may confer its competence by regulating the receptor action at the target DNA sequences\textsuperscript{16}.

\textbf{E93}: is a critical regulator of 20E-triggered PCD or programmed cell death, important for maintaining tissue homeostasis during insect metamorphosis. The \textit{E93} mutants displayed persistent salivary glands at 24 hrs APF and ectopic expression of E93 restored the necessary cell death response in the mutant background. E93 is a novel nuclear protein with no matches in the sequence database and is expressed in doomed larval cells foreshadowing their histolysis by a 20E-mediated PCD. For example, \textit{E93} is expressed in midguts of the prepupae prior its histolysis by PCD but not in the salivary glands or the diploid cells forming the adult midgut epithelium. In addition, E93’s expression is detectable in a subset of cells in the developing eye and CNS but not in the leg or wing imaginal discs during the prepupal development. Following the pulse of 20E at 10-12 hrs APF, E93 is expressed in the salivary glands triggering its histolysis. In addition to the block in salivary gland histolysis, defective body shortening and anterior spiracle eversion also characterize the \textit{E93} mutants. Expression of a number of 20E regulated genes and PCD genes were affected in the mutant pupae. This observation was in alignment with the immunostaining result in which E93 bound to specific sites containing 20E regulated genes and PCD genes in the larval salivary gland polytene chromosomes\textsuperscript{8,17}.

In addition to the 20E-regulated genes discussed in this section, many other 20E
response genes have been identified and their functions analyzed by generating mutant alleles. For a clear understanding, a graphical representation depicting the transcriptional and biological programs in response to late larval and prepupal 20E pulses is given below. The figure is adapted from a report by Lam et al in 1999\textsuperscript{11}.

Figure 1: The 20E directed major developments at the onset of metamorphosis are addressed with respect to time at the top and bottom of the figure. Solid orange boxes depict transcription of 20E regulated early genes and the competence factor. The solid yellow boxes denote the 20E peaks triggering the larval to prepupal and prepupal to pupal transitions respectively.
Another gene that is required for larval salivary gland histolysis is Mdh2 (CG7998). It is expressed in the pupal phase but whether or not its expression is driven by 20E titers is unknown currently. It codes for a predicted malate dehydrogenase and localizes to mitochondria. Mdh2 mutants display no obvious morphological defects except for a block in salivary gland histolysis. Expression of key 20E inducible cell death regulators like rpr, hid and dronc was normal in the mutant salivary glands. The mutants instead, suffered from an impaired TCA or citric acid cycle and accumulated late-stage TCA intermediates at 12 hrs APF. In addition, ATP levels were also significantly reduced in the mutants. These observations suggested that the block in salivary gland histolysis in the Mdh2 mutants was probably triggered by an energy deficit\textsuperscript{18}.

*Drosophila melanogaster* constitutes an ideal model system for studying the phenomenon of development\textsuperscript{19}. Its developmental stages are morphologically distinct and are comparable to human development, before and after puberty. These stages have been studied for close to a century and as such, a huge volume of information exists to support new research.

**Polytene chromosomes in *Drosophila***

Polytene chromosomes of *Drosophila melanogaster* have been studied extensively as a model for eukaryotic interphase chromatin. They are characterized by alternate appearance of bands (dark stripes) and interbands (light stripes) when viewed under phase contrast microscope. Such alternating banding pattern is
highly reproducible and has been compiled into a chromosome map. Because of the ease of manipulation, existence of a chromosome map and a huge volume of information available on its structure and function, salivary glands have been used to investigate multiple aspects of gene structure and expression (mapping of individual genes, analyzing their expression as well as locating sites of protein accumulation). Examination and analysis of numerous Hoechst-stained polytene chromosome squash preparations by phase contrast- or fluorescent microscopy-imaging has led to the description of the more densely packed chromatin as bands and the more dispersed chromatin as interbands. Numerous studies have shown interband-specific localization of RNA polymerase II in addition to other factors of the transcription-machinery. Also, the interbands and puffs have been found to be sites of high gene-expression. The generally accepted notion is that, the interbands are associated with gene expression and its decondensed morphology facilitates the process in many ways including an easy access to the transcription factors\textsuperscript{20}. The giant salivary gland chromosomes are generated through a process of “endoreduplication”, in which multiple rounds of DNA replication occur without subsequent mitosis and cytokinesis. In addition to salivary glands, many larval tissues are polytenized but to a lesser extent than the salivary glands. This difference of polytenization has been attributed to a relatively early onset of endoreduplication in the salivary glands compared to other tissues. The replicated DNA, in absence of the subsequent process of strand separation of mitosis, are held in register and aligned side-by-side. Because of this, it is easy to dissect,
experimentally manipulate and visualize the giant polytene chromosomes under microscope\textsuperscript{20,21,22,23}.

Endoreduplication is evolutionarily conserved and employed by specialized tissues to trigger growth by an increase in cell size. In addition to salivary glands, endoreplication has been detected in germline-derived nurse cells. The nurse cells form an interconnected network or cyst and share their cytoplasm with the growing oocyte to supple maternal products such as mRNA and proteins. Due to absence of zygotic transcription in the early stages of embryogenesis, this maternal supply of products from the polyploid nurse cells is crucial for growth and development\textsuperscript{24,25}.

**Euchromatin and heterochromatin**

Just like any interphase chromatin, the salivary gland polytene chromosomes can be subdivided into zones of heterochromatin and euchromatin, mostly depending on the level of compaction and transcriptional activity. Parts of arms from polytene chromosomes are generally considered to be euchromatic if they are gene rich, less condensed and transcriptionally active. Parts of arms from polytene chromosomes (chromocenter, telomeres) are considered to be heterochromatic if they are gene-poor, highly condensed, and transcriptionally repressed\textsuperscript{26}. Heterochromatin can be further sub-divided into constitutive and facultative heterochromatin. Constitutive heterochromatin is made up of repetitive DNA and
“constitutively” condensed. On the other hand, the term, “facultative heterochromatin” is associated with euchromatic DNA that has transformed into a heterochromatin-like state. Such transformation may occur in response to environmental changes induced by alterations in histone modifications\textsuperscript{27,28}.

During the process of endoreduplication, the differences between euchromatin and heterochromatin become very conspicuous. While the euchromatin arms proceed through about 10 DNA doublings resulting in $2^{10}$ or 1024 strands, the heterochromatic regions, especially the chromocenter, undergo little or no replication.

Both heterochromatin and euchromatin are critical for maintaining genomic integrity. Spread of repressive chromatin marks from heterochromatin to adjacent euchromatin trigger modifications of gene expression programs and is often implicated in malfunctioning of cells. Position-effect variegation (PEV) studies in \textit{Drosophila} have analyzed the functional consequences of such spread in the context of transcription of the \textit{white} gene. The \textit{white} gene is located near the tip of the X chromosome and is expressed under normal/control conditions to form the red pigments of the \textit{Drosophila} eyes. However, if the \textit{white} gene is placed in a new position adjacent to the pericentromeric heterochromatin by chromosomal rearrangement, it undergoes variable inactivation during development and is silenced in a certain fraction of cells and expressed in others. This variable silencing of the \textit{white} gene generates the “mosaic-eye” phenotype with red
pigmentation (where the gene is expressed) and patches of white (where the gene is silenced)\textsuperscript{29,30}. As will be discussed in the next sub-heading, gene silencing is regulated by a plethora of factors, including epigenetic histone modifications. One modification associated with gene silencing is histone methylation. Histone methylation is reported to silence gene expression by 1) antagonizing euchromatic markers, such as phosphorylation and acetylation\textsuperscript{31,32} and 2) acting as a docking site recruitment of heterochromatin-assembly protein, such as HP1 and Su(var)3-9\textsuperscript{33,34}.

**Histone modifications**

The amino-terminal tails of core histones are sites of extensive modifications like acetylation, phosphorylation, methylation among others. Such modifications are critical regulators of chromatin structure and function with the potential of influencing gene expression, recombination and repair\textsuperscript{35}. For example, acetylation of histone-lysines neutralizes their positive charges and causes the negatively charged phosphate backbones to repel one another and open up\textsuperscript{36}. Opening up or loosening of the chromatin is in general associated with gene expression and in the case of polytene chromosomes, mapped to the interbands or puffs. In this study, cellular growth and division and gene expression have been studied in the context of histone phosphorylation and histone methylation. These two modifications will be discussed here briefly.
**Histone phosphorylation**

The histone tails can be phosphorylated and dephosphorylated by kinases and phosphatases respectively. Phosphorylation of histones can occur on serine, threonine and tyrosine. The H3S10ph mark has been extensively studied with respect to chromosome compaction and gene expression. Studies have uncovered two seemingly contradictory sides of this modification: a) Association with loosely packed chromatin and active gene transcription during interphase b) association with chromosome condensation during mitosis\(^{37}\). H3S10ph during interphase does not affect the entire genome but regulates only a small subset of transcriptionally active genes. For example, the H3S10ph mark of the *Fos* gene is elevated during mitogen activated gene expression\(^{38}\) in interphase. In *Drosophila*, the H3S10ph mark is elevated at the interbands and puffs, sites of active gene expression on polytene chromosomes. During mitosis, the ser10 residue of H3 is phosphorylated in all eukaryotes. PH3 or phosphohistone-H3 has been routinely used as a marker of Mitosis. PH3 is detected on only pericentromeric chromatin in late G2. PH3 spreads along chromosome arms as mitosis progresses and the event is complete by the end of prophase. At the end of mitosis, H3 is dephosphorylated\(^{38,39}\).
Histone methylation

Histones can be methylated or de-methylated on lysines by methyltransferases and demethylases. Histone methylation has been associated with both transcriptional activation and inactivation and as such, its interpretation in the context of gene expression has not been straightforward\(^{36}\). One to three methyl groups can be added to lysine residues at different positions on the histones. Both the degree of methylation as well as the specific lysine residue methylated can have a bearing on the resultant gene expression pattern\(^{40,41}\). Briefly, enrichment of H3K4me, H3K36me or H3K79me is reported on transcriptionally active chromatin\(^{42}\). In contrast, enrichment of H3K9me, H3K20me or H3K27me has been correlated with repressed/silenced chromatin\(^{43}\). In addition, combination of different histone-lysine methylation markers may have effects different from that of individual markers. For example, both H3K4me3 and H3K27me3 could be detected on genes involved in stem cell development and are believed to keep the genes poised for activation as and when required\(^{44}\).

The consequences of epigenetic histone modifications are diverse and their interpretation complex. The dynamic epigenetic state of chromatin governed by diverse histone modifications may regulate the "euchromatic" (on) or "heterochromatic" (off) state of the chromatin\(^{45}\). The epigenetic histone modifications in interphase have been extensively studied in *Drosophila salivary*
glands and those in mitosis have been analyzed in the neuroblasts from larval brains.

**The brain and neuroblasts**

While increase in cell size occurs with increase DNA ploidy in tissues like salivary glands undergoing endoreduplication, the fruitfly also contains cells that undergo mitosis to increase the cell number rather than cell size. These cells are present in tissues destined to form the adult-specific organs and appendages like the imaginal discs, larval neuroblasts, germ cells and the abdominal histoblast nests\(^24\). In this study the mitotic cells examined are the larval neuroblasts and in this section, their development will be discussed briefly.

The brain of insects (fruitfly) is formed by a unique population of cells with stem-cell like features, the “neuroblasts”\(^46,47\). In an early embryo, the neuroblasts delaminate from the neuroectoderm and form a layer of large, rounded cells. Soon these cells start proliferating in a stem-cell like manner. While most of the embryonic cells undergo symmetrical cell divisions, the neuroblasts undergo asymmetrical cell division\(^48\) generating one large daughter cell, also called the “neuroblast” and one small daughter cell, called the GMC or “ganglion mother cell”. These two daughter cells have different developmental fates. While the neuroblasts continue to divide for several rounds in the aforementioned stem cell like manner, the GMC usually divides only once after its birth, forming 2 daughter cells that become post-mitotic and differentiate into neurons and glial cells.
respectively\textsuperscript{48,49}. The last cycles of primary neuroblast division are usually detectable at embryonic stages 14 and 15, beyond which only the GMC divisions are detectable for another 2 to 3 hours. The primary neuroblasts then shrink in size, stop dividing and become quiescent. A subpopulation of the neuroblasts (e.g. mushroom body neuroblasts) however, evades the mitotic arrest and constantly divides throughout the larval stages into the pupal stage\textsuperscript{50,51}. The first wave of neurogenesis is completed approximately at the end of embryonic stage. It generates all the neurons required to form the larval CNS but only 10\% of the cells required to form the adult CNS. In a mechanism not very well understood, during the late 1\textsuperscript{st} instar or early 2\textsuperscript{nd} instar, the quiescent neuroblasts become mitotically active such that the full complement of neuroblasts now proliferates throughout the 3\textsuperscript{rd} larval instar into the pupal stage. This is the second wave of neurogenesis that generates 90\% of the neurons of the adult CNS. At this point, the second wave of neurogenesis stops and neuroblasts exit cell cycle at different time points\textsuperscript{47}.

Neuroblasts are generally classified into 5 sub-types and can be detected in different parts of the larval brain\textsuperscript{52}.

a. Type IA neuroblasts that are eliminated during embryonic stage by apoptosis generate small lineages that ultimately constitute the abdominal region of the VNC or the ventral nerve cord.

b. Type IB neuroblasts continue to divide till the pupal stage and undergo the 2 waves of neurogenesis with a quiescent stage in between the 2 waves.
Unlike the type IA neuroblasts, the type IB neuroblasts are not eliminated by apoptosis but by a specific form of cell cycle exit in the pupal stage. They constitute the thoracic neuroblasts. Both type IA and type IB neuroblasts undergo asymmetric cell division, forming a GMC and a self-renewing neuroblast.

c. The mushroom body neuroblasts, found in the brain lobes, generate large postembryonic lineages by continuous asymmetric divisions without undergoing quiescence.

d. Type II neuroblasts are detected in the medial part of the central brain. These neuroblasts undergo asymmetric divisions to generate neuroblasts and self-renewing non-neuroblast precursors called INP (Intermediate neural precursor). The INP divides to form a series of GMCs, which in turn divides to form neurons and glial cells.

e. The optic lobe neuroblasts arise during the larval period by asymmetric divisions and are located in the brain lobes.

It is important to bear in mind that neuroblasts may spend variable lengths of time in the quiescent stage before the 2nd wave of division in the larval stage starts and hence, not all of them are active at a given time. A multitude of factors regulate the time-span of neuroblast quiescence, re-entry into the cell cycle, and the number of mitotic cycles to ensure the proper development of brain before the larva progresses into the pupal stage and ultimately an adult fly53–55.
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CHAPTER 3. dASCIZ, A CHROMATIN ASSOCIATING PROTEIN, CONTRIBUTES TO FRUITFLY DEVELOPMENT BY REGULATING THE EXPRESSION OF ECDYSONE REGULATED GENES.

(A paper to be submitted)

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Summary

Atmin, initially identified to be an ATM-kinase interacting stress response protein, was later shown to be crucial for embryonic development. It was reported to regulate kidney organogenesis, lung morphogenesis and ciliogenesis by acting as a transcription factor and in a manner independent of its role in stress resolution pathways. However, its role in post-embryonic development could not be deduced as the Atmin mutants described thus far, are embryonic lethals. In this study, we identified and characterized an EP-element insertion line EP(3)3709 as a dASCIZ mutant. EP3709 homozygotes never eclosed as adults but survived till the pupal stage, displaying severely impaired metamorphosis. Mutant pupae displayed little or no morphogenesis of adult head, wings, legs and abdomen and persistent salivary glands at 24 hours APF, implicating a defective ecdysone signaling. Expression of a number of genes central to the ecdysone-signaling cascade, was found to be aberrant in mutant pupae. The size of the brain in the preceding 3rd
instar larval stage was smaller and the mutant larvae appeared more transparent than the WT controls. The EP-element induced lethality was rescued by ectopic expression of a transgenic reporter protein dASCIZ-HA-mCitrine indicating \textit{EP(3)3709} is a true mutant allele of \textit{dASCIZ}. The dASCIZ-fusion protein associated with chromatin and localized mainly to interbands on polytene chromosomes with an extensive overlap of signal with RNApolIIser5. In doing so, it exhibited two types of staining patterns: 1) a widespread occupancy of the interbands at relatively low intensity signal, referred to as the global staining; 2) a selective occupancy at a subset of those interbands, including puffs at an elevated intensity, referred to as discrete staining. dASCIZ-HA-mCitrine also displayed a characteristic staining pattern on polytene chromosomes before and after heat shock treatment. When analyzed on polytene chromosomes immediately after heat shock (heated in a water bath for 37°C for 1 hour), the dASCIZ-HA-mCitrine signal from discrete staining was undetectable but recovered after extended period of incubation at room temperature. Together our results identify a role for \textit{dASCIZ} in fruitfly development in the post-embryonic stages of the last larval instar and metamorphosis, and describe its chromatin association properties, including a characteristic staining pattern before and after heat shock.

\textbf{Introduction}

A transcription factor may be involved in different signaling pathways that may or may not be linked to one another to maintain multiple physiological processes.
Genome wide profiling made possible by advanced molecular and computational techniques has often allowed the understanding of the multiple biological networks a transcription factor may be involved in. For example, proteins of the Myc family were initially reported to control the expression of a battery of genes involved in cell growth, cell death and cell cycle by acting as a transcription factor\textsuperscript{1,2,3}. Recent reports have shown that the Myc transcription factors are also involved in regulating the initiation of DNA replication and protein expression through mRNA translation\textsuperscript{4,5,6}. These observations highlight their roles in processes beyond cell cycle and apoptosis. Another extensively studied protein with diverse functions is Sp1. Previous studies described Sp1 as a transcription factor involved in regulation of expression of a multitude of genes implicated in cell growth, differentiation, apoptosis, immune response, and angiogenesis in response to physiological and pathological stimuli\textsuperscript{7–10}. Later, a role of Sp1 in DNA repair was predicted when it was identified to be a substrate for ATM kinase in response to stress stimuli like IR (ionizing radiation) and H\textsubscript{2}O\textsubscript{2} (Hydrogen Peroxide)\textsuperscript{11}. Subsequent studies showed that Sp1 gets phosphorylated on serine 101 and localizes to ionizing radiation-induced DSBs (Double stranded breaks) with γH2A\textsubscript{x} (phosphorylated histone variant) and MRN complex (Mre11, Rad50, and Nbs1). Using laser microirradiation and ChIP (chromatin immunoprecipitation), it was shown that within 7.5 mins from induction of DNA damage, Sp1 localized to a site within 200 bps from the DSBs and remained there.
for a minimum of 8 hrs. RNAi of Sp1 impaired repair of site-specific DNA breaks at a magnitude comparable to that of NBS1 RNAi. Interestingly, the N-terminal 182-amino-acid peptide of Sp1 (without the DNA binding ZNFs of the C-terminus) was sufficient for phosphorylation by ATM kinase, localization to the DSBs, and rescue of DNA damage repair defect of Sp1 RNAi. These observations described a role of Sp1 in DNA damage repair that is independent of its function as a transcriptional factor. Hence, an understanding of the multifarious nature of a protein will help us appreciate its importance in the context of maintaining gross organismal physiology. Such studies may also trigger the discovery of new proteins expanding our knowledge of the proteome.

ATM/ATR-substrate CHK2-interacting Zn$^{2+}$-finger protein or ASCIZ was originally identified as a novel protein involved in Rad51 foci formation in response to damage by MMS but not IR. Subsequent studies showed it interacted with the checkpoint kinase ATM (ataxia telangiectasia mutated) and based on this interaction came to be called Atmin or ATM-kinase interactor protein. Atmin orchestrates an ATM-kinase dependent but NBS1 independent repair response during non-IR stresses like hypotonic stress or oxidative stress. Atmin is a protein of 823 amino acids with a ZnF domain at the N-terminus and an SCD in the C-terminus. It is sometimes considered to be a mirror image of the transcription factor Sp1. In addition to its role in stress response pathways, Atmin has been reported to play a central role in promoting development of B-cells from the pro-B cell stage and morphogenesis of organs like lungs and...
kidneys during embryonic development by mainly acting as a transcription factor of *Dynll1* (Dynein light chain) that is independent of its function as a DNA damage repair protein\(^\text{17–19}\).

In 2014 Zaytseva *et al.*, reported the identification of *CG14962* as the *Drosophila* ortholog of *ASCIZ* or *Atmin*\(^\text{20}\). *CG14962* or *dASCIZ* encodes a protein of 388 amino acids with Zinc fingers in the N terminus and an SCD (1 SQ and 7 TQ) in the C terminus. Localized *dASCIZ* knockdown in the posterior compartment of the wing imaginal discs using en-Gal4 driver affected tissue development in the wing discs. Analysis of the defects revealed *dASCIZ* acted as a novel regulator of mitosis by transcriptionally activating *Dynll1* expression\(^\text{20}\). Tissue specific loss of *dASCIZ* was described in this report since the global RNAi knockdown led to 1\(^{\text{st}}\) instar larval lethality with 100% penetrance.

The null alleles and the point mutants of *Atmin* described in murine models are all embryonic lethals precluding any analysis of its roles in post-embryonic development. Also, the global RNAi knockdown in the fly model led to 100% 1\(^{\text{st}}\) instar lethality preventing analysis of its roles in later stages of development like the larval instars or metamorphosis\(^\text{16,18,19,20}\).

In this study, we explored *dASCIZ*’s role in post-embryonic fruitfly development, specifically in metamorphosis and 3\(^{\text{rd}}\) larval instar. The extensively studied 3\(^{\text{rd}}\) instar larval stage as well as the well-characterized events of metamorphosis make *Drosophila* an excellent model for this study\(^\text{21,22,23}\). We identified and characterized a P-element insertion mutant *EP(3)3709* as a *dASCIZ* mutant and
used it as a platform to study its contributions in *Drosophila* development. To generate another mutant allele of *dASCIZ*, the EP element was mobilized by Δ2-3 transposase resulting in imprecise excision. The putative imprecise excision mutant *IP3709/IP3709* recapitulated some of the defects of the *EP(3)3709/EP(3)3709* like pupal lethality and impaired metamorphosis. In addition, we visualized the interactions of dASCIZ-fusion protein with chromatin in vivo and reported a characteristic staining pattern on polytene chromosomes before and after heat shock treatment.

**Experimental Procedures**

**Drosophila strains**

Fly stocks were maintained at 25°C according to standard protocols. Canton S was used for control or wild type preparations. Act5C-Gal4 driver, Sgs3-GAL4 driver as well as the *EP(3)3709* mutant line were obtained from the Bloomington Stock Center. The *w; Δ2-3/TM2Ubx* stock was the generous gift of Dr. Linda Ambrosio. Gateway cloning was used to clone the full-length *dASCIZ-mCitrine* cDNA into the destination vector pPFHW with 3xHAtags in the N terminus (Construct henceforth referred to as: dASCIZ-HA-mCitrine). Transgenic UAS-*dASCIZ-HA-mCitrine* lines were generated by P-element-mediated transformation by BestGene and suitably mapped.
**Polystene chromosome squash**

Polystene chromosome squash was performed using either 1 min or 5 mins fixation protocols and suitably immunolabeled. Primary antibodies used for squash included mouse anti-HA (Roche) and RNAPolIIser5 (Covance). DNA was stained with Hoechst 33258 (Molecular Probes) in PBS for visualization. The appropriate species- and isotype- specific secondary Abs used were TRITC-conjugated anti Mouse IgM and FITC-conjugated Goat anti-Mouse Ig1 (Southern Biotech) at 1:200 dilution to detect the primary antibody labels. Mounting was done in 90% glycerol containing 0.5% n-propyl gallate. The preparations were examined using epifluorescence optics on a Zeiss Axioskop microscope and images were captured and digitized using a cooled Spot CCD camera. Images were imported into Photoshop for pseudocoloring, image processing and then merged. In some images non-linear adjustments were made to the channel with Hoechst labeling for optimal visualization of chromosomes.

**Western blotting analysis**

Protein extracts were prepared from salivary glands or brains dissected from third instar larvae and homogenized in a buffer containing: 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 10 mM EDTA, 1 mM EGTA, 0.2% Triton X-100, 0.2% NP-40, 2 mM Na3VO4, 1 mM phenylmethylsulfonyl fluoride, 1.5 1g/ml aprotinin. Protein separation by SDS-PAGE was performed according to standard procedures. Bio-Rad Mini PROTEAN III system was used for gel-running. 0.2 μm nitrocellulose
and Transfer-blot Turbo (Biorad) were used for electroblotting and electro-transfer under semi-dry conditions respectively. Primary labels used were: Mouse anti-tubulin (sigma), Mouse anti-HA (Roche), Mouse anti-lamin DM0 (Developmental Studies Hybridoma Bank, Iowa City), Rabbit anti-H3S10ph (Cell Signaling), goat anti-histone H3 (Santa Cruz Biotechnologies), Rabbit anti-dASCIZ 1183 Ab (a generous gift of Dr. Jörg Heirhorst, St. Vincent’s Institute of Medical Research, Australia), all used at 1:1000 dilutions. Secondary labels such as HRP conjugated anti-mouse and anti-rabbit (BioRad) secondary Abs were used at 1:2000 dilution. Antibody labeling was visualized using chemiluminescent detection methods (SuperSignal West Pico Chemiluminescent Substrate, Pierce). The immunoblots were digitized using a flatbed scanner (Epson Expression 1680).

**Staging and characterization of pupae**

Pupae were synchronized at the white prepupal stage as T=0 and incubated to follow development during metamorphosis and analyzed in accordance with Bainbridge and Bownes²⁷.

**Acridine orange staining of larval brains**

Acridine orange or AO staining was performed as described in *Drosophila* protocols by Sullivan *et al*²⁸. Brains were dissected in Ringer’s solution from wandering 3rd instar larvae, incubated in in $1.6 \times 10^{-6}$ M solution of Acridine orange (Sigma) for 5 mins and again rinsed briefly in PBS. The samples were mounted in Ringer’s solution and imaged immediately.
H3S10ph staining of larval brains

The procedure for immunostaining of larval brains was adapted from a protocol established by Wu et al in 2006\textsuperscript{29}. Briefly, the larval brains were dissected from wandering 3\textsuperscript{rd} instar larvae, fixed in 4\% paraformaldehyde (PFA), permeabilized in 0.04\% PBST and blocked in 1\% Normal goat serum or NGS. Primary Ab labeling was done with Rabbit H3S10ph (Cell Signaling) at a dilution of 1:100 at 4°C for 48 hours, followed by three 20 minute washes in PBST. Secondary Ab labeling was done with Donkey anti-Rabbit TRITC (Jackson Immuno Research) at a dilution of 1:200 at 4°C for another 48 hours, followed by three 20 minute washes in PBST. Brains were then mounted and imaged as previously described.

Bromodeoxyuridine labeling of larval brains

The staining protocol was adapted from Truman et al., 1988 and Bolkan et al., 2007\textsuperscript{30,31}. Brains dissected from wandering 3\textsuperscript{rd} instar larvae were incubated in Shields and Sang M3 insect medium (Sigma) containing 1 mg/ml 5-bromo 2-deoxyuridine (BrdU; Sigma). Incubation was allowed for 1 hr at room temperature (RT). Brains were then fixed, permeabilized in 0.04\% PBST and treated with 2 N HCl in PBST for 30 mins at RT. After 2 washes in 0.02\% PBST, 15 minutes each, brains were incubated in 1\% NGS for 1 hr at 4°C. For primary Ab labeling, brains were labeled with mouse anti-BrdU (Developmental Studies Hybridoma Bank, Iowa City, IA)\textsuperscript{31} at a dilution of 1:150 for 36 hours at 4°C. After three 20-min washes in PBST, secondary Ab labeling was done with FITC conjugated Goat
anti-Mouse IgG (Southern biotech) for another 36 hours at 4°C. Following another three 20-min washes in PBST, brains were mounted and imaged as previously described.

RNA isolation and gene expression analysis

RNA was isolated from wandering 3rd instar larvae or staged pupae using UltraClean® tissue and cells RNA isolation kit (MO Bio laboratories, Inc.) according to manufacturer’s instructions. 1ug of RNA was DNase (Invitrogen) treated and converted into 1st strand of cDNA using RT-PCR SuperScript III kit (Invitrogen) according to the manufacturer’s instructions. For determining changes in gene expression in wandering 3rd instar larvae, quantitative PCR was carried out with the cDNA template using Brilliant® II SYBR Green QPCR Master Mix (STRATAGENE) in an Mx4000 (STRATAGENE) PCR machine. Cycling parameters used were 10 min at 95°C, followed by 40 cycles of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C. Fluorescence intensities were plotted against the number of cycles using an algorithm provided by Stratagene. Relative abundance in transcripts was calculated using the $2^{-\Delta\Delta CT}$ or Livak method\textsuperscript{32}. For analysis of changes in ecdysone signaling in staged pupae, RT-PCR was conducted under thermocycler conditions as: Initial denaturation at 94°C for 5 mins followed by 94°C for 1 min, 60°C for 1min, 72°C for 1 min, and a final 72°C elongation period (10 min).
For ecdysone response, following number of cycles was used for different genes

<table>
<thead>
<tr>
<th>Gene type</th>
<th>E74</th>
<th>E93</th>
<th>βFtz-f1</th>
<th>ECR</th>
<th>BR-C</th>
<th>Mdh2</th>
<th>dASCIZ</th>
<th>RP49</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cycle number</td>
<td>29</td>
<td>29</td>
<td>29</td>
<td>28</td>
<td>28</td>
<td>31</td>
<td>35</td>
<td>22</td>
</tr>
</tbody>
</table>

For both the quantitative and semi-quantitative PCRs, results represent the average of 3 or more biological replicates.

The following sets of primer pairs were used

**EcR** Forward 5’ - GGAGAACCAATGTGCGATGA- 3’

**EcR** Reverse 5’ - AAGGAAGGTATATTGCGGC - 3’

**BR-C** Forward 5’ - ACCCAATCGCTCCACATCCG - 3’

**BR-C** Reverse 5’ - GTTGCTGCCACTGCAAACG - 3’

**E93** Forward 5’ – AGAACGCAGTTGCTGAAGAAT-3’

**E93** Reverse 5’ – GATTGCTCTGGCTGATCTCC-3’

**βFTZF1** Forward 5’ – TGGCGTACTTTTAGCGTCCT -3’

**βFTZF1** Reverse 5’ – AATACAAGAATCGATCTTCAAGTG -3’

**74EF** Forward 5’ - AACGTGCAATCTCTTTGAAAGG - 3’

**74EF** Reverse 5’ - AGATAAAGGTACTTTAGAGAAAGCAATG - 3’

**MDH2** Forward 5’ – CATATCGACACCAAGGAGAGAAGG - 3’

**MDH2** Reverse 5’ – GTGGAGACTCCGAACAGAGG - 3’
**Results**

**Isolation and analysis of dASCIZ mutants**

We identified CG14962 as the *Drosophila* ortholog of ATM-kinase interactor protein or Atmin by sequence homology searching and named it “Digitor”. The amino acid sequence of Digitor is closely related to that of human Atmin as shown in the MUSCLE alignment in figure 1 A. A total of 257 residues are either identical (111 residues, 28.6 %) or similar (146 residues, 37.6 %) between the 2 proteins. While this work was in progress, Zaytseva *et al* in 2014\(^2\) published a report, also identifying CG14962 to be the *Drosophila* ortholog of Atmin (or ASCIZ) and named it dASCIZ. Henceforth in this thesis, CG14962 will be referred to as dASCIZ. *dASCIZ* is located at the cytological position 63B8. To study the function of dASCIZ *in vivo*, we searched the flybase for P-element

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**CG32280** Forward 5’- ATGAGTAAACCTGGCAGTGCTCC -3’

**CG32280** Reverse 5’ - CTATCGACGGTATCGCCCCA -3’

**dASCIZ** Forward 5’ - ATGCACAGCGAAAAACACACG -3’

**dASCIZ** Reverse 5’ - CTTGTAGTTTCTTGCCAGGTGGCTC -3’

**Cut-up** Forward 5’ - ATCGAATTTCATGTCTGATCGCAAGGCCG -3’

**Cut-up** Reverse 5’ - ATCTCTAGATTAACCAGCTCTTTAAACAGTAAAATAGCC -3’

**RP-49** Forward 5’ - ATGACCATCCGCCCAGCATA C -3’

**RP-49** Reverse 5’ – GAATCCGGTGGCAGCATG -3’
insertions either in the vicinity or inside the open reading frame of \textit{dASCIZ}. \textit{EP(3)3709} (Flybase ID FBst0017158) was identified as a putative \textit{dASCIZ} mutant line that contained a P element insertion 27 base pairs downstream of the ATG initiating codon (http://www.ncbi.nlm.nih.gov/nucgss/AQ073134). Considering \textit{dASCIZ}'s partial overlap with the oppositely transcribed \textit{CG32280} and the position of P element insertion (figure 1 B), we speculated that, such an insertion might disrupt the expressions of both \textit{dASCIZ} and \textit{CG32280}. To test this, we checked the relative levels of \textit{dASCIZ} and \textit{CG32280} messages by RT-qPCR in the WT and \textit{EP(3)3709/EP(3)3709} homozygotes using whole animal RNA extracts from wandering 3\textsuperscript{rd} instar larvae. In addition, we also examined the \textit{Cut-up} messages from the putative mutant. \textit{Cut-up} has been established to be an evolutionarily conserved transcriptional target of \textit{dASCIZ}. Since a perturbation in an inducer/activator gene expression has been seen to affect target gene expression, a decrease, should there be any of \textit{dASCIZ} messages in \textit{EP(3)3709/EP(3)3709} should effect a corresponding decrease in \textit{Cut-up} messages. As shown in figure 1 C, the P element insertion clearly affects \textit{dASCIZ} expression and its transcriptional target \textit{Cut-up} without any major perturbation of \textit{CG32280} expression. For this reason, \textit{EP(3)3709/EP(3)3709} line was used in subsequent experiments of mutant analysis.

The P-element mediated insertional inactivation of \textit{dASCIZ} induced lethality at the pupal stage with a complete failure of eclosion. Percentage eclosion was scored as
the number of empty pupal cases counted and expressed as a percentage of total number of pupae counted (figure 1 D).

**Generation of transgenic flies expressing dASCIZ-HA-mCitrine reporter protein**

Transgenic flies were generated expressing a dASCIZ fusion protein under UAS-GAL4 control. dASCIZ with mCitrine cDNA at its C-terminus was cloned into the destination vector pPFHW with a 3xHA tag in its N terminus by Gateway Cloning and used to generate transgenic flies. The resultant reporter protein will be henceforth referred to as dASCIZ-HA-mCitrine. Using different Gal-4 drivers, its expression was tested in different organs dissected from wandering 3\textsuperscript{rd} instar larvae. When driven by the ubiquitous Act5C-Gal4 or Actin-Gal-4 driver, live dASCIZ-HA-mCitrine expression could be detected in brains, salivary glands and wing discs (figure 2 A-C). When driven by the salivary gland specific Sgs3-Gal4 driver, live expression could be detected in salivary glands only (figure 2 D). Expression of the fusion protein was further verified in protein extracts prepared from salivary glands by western blotting (figure 2 E). A mouse monoclonal anti-HA Ab was used for detecting the fusion protein. Though its predicted size is \(~70\) kd, dASCIZ-HA-mCitrine was found to migrate at a much higher position (approximately 116 kd) on a reducing protein gel. An identical migration pattern was produced when probed by 1183 rabbit antibody (figure 2 E). Thus, the band recognized by the anti-HA is specific for dASCIZ-HA-mCitrine. Inspection of the amino acid sequence of dASCIZ revealed the presence of 27 proline residues
Proline is known to introduce kinks in the backbone of a protein causing it to migrate slower than expected in SDS gels\textsuperscript{34}. In addition, it has 1 SQ and 7 TQ motifs (figure 2 F). SQ/TQ motifs are sites of myriad post-translational modifications like phosphorylation, also implicated in retardation of gel migration\textsuperscript{35,36}. It is possible that the combination of the aforewritten factors severely retard the migration of dASCIZ-HA-mCitrine resulting in a much higher observed molecular weight than predicted.

**Rescue of** \textit{EP(3)3709/EP(3)3709} **lethality by ectopic expression of the transgene**

To verify that the pupal-lethal phenotype of the \textit{EP(3)3709/EP(3)3709} was indeed caused by disruption of \textit{dASCIZ}, we attempted to rescue it by ectopically expressing the transgenic reporter protein dASCIZ-HA-mCitrine. To that end, we generated 2 fly lines. One line carried the reporter-transgene on the second and the mutant allele on the third \textit{(dASCIZ-HA-mCitrinew+/CyOy+/EP(3)3709w+/TM6b,Sb,Tb,e\textsuperscript{3}).} The other line carried the driver on the second and the mutant allele on the third \textit{(Act5C-Gal4w+/CyOy+/EP(3)3709w+/TM6b,Sb,Tb,e\textsuperscript{3}).} Crossing these 2 lines generated a homozygous mutant background on the 3\textsuperscript{rd} chromosome while simultaneously allowing us to drive the expression of dASCIZ-HA-mCitrine by the Act5C-Gal4 driver from the second chromosome. Genotype of the line is: \textit{dASCIZ-HA-mCitrinew+/Act5C-Gal4w+/EP(3)3709w+/EP(3)3709w+}. Percentage eclosion of this line was scored and rescue percentage was calculated to be 23 (figure 3 C).
Live dASCIZ-HA-mCitrine expression could also be detected in the ovaries of the rescued flies (figure 3 A-B). The UAS-dASCIZ-HA-mCitrine cDNA construct alone, in the absence of any GAL4 driver, was able to rescue the lethal phenotype of EP(3)3709/EP(3)3709 to very small extent (7%) (figure 3 C). Instances of such non-driven rescue have been previously reported in literature\textsuperscript{37}. It likely results from “leaky” expression and is contingent on the site of insertion of the transgenes and the number of transgenes present\textsuperscript{37}. 0% rescue was recorded in the Act5C-Gal4w+/CyOy+;EP(3)3709w+/ TM6b,Sb,Tb,e\textsuperscript{8} stocks (figure 3 C) indicating that the observed rescue was due to expression of the transgene in the mutant background. These results suggested that EP(3)3709 is a true mutant allele of dASCIZ.

**dASCIZ-HA-mCitrine binds to open regions of the chromatin**

dASCIZ has 4 ZNFs in the N-terminus. One of the most commonly described roles for this class of proteins is chromatin binding\textsuperscript{38}. We investigated the association of dASCIZ-HA-mCitrine on polytene chromosomes of salivary glands dissected from transgenic wandering 3\textsuperscript{rd} instar larvae in both fixed and live preparations. Polytene chromosome squash preparations were triple labeled with anti-HA (for staining dASCIZ-HA-mCitrine), actively transcribing RNA polymerase II\textsuperscript{ser5} and Hoechst (for staining DNA) (figure 4 A). Previous studies have shown that Hoechst staining is weak or absent in the interbands or open regions of the DNA and bright in the bands or condensed regions of the DNA\textsuperscript{24,39}. 
dASCIZ-HA-mCitrine localizes primarily to regions of little or no Hoechst staining indicating a widespread occupancy of the interbands. In addition, it shows an extensive overlap of its signal with the actively transcribing RNA polIIser5 present almost exclusively in interbands and puffs (figure 4 A-D). We refer to this staining pattern of the fusion protein as global staining. Interestingly, its signal is significantly increased at some sites of occupancy compared to others on polytene chromosomes (arrows in figure 4 B). We refer to this increased signal at those sites as discrete staining. Interbands have been proposed to be sites of active gene expression. Hence, dASCIZ-HA-mCitrine’s broad association with the polytene chromosome interbands coupled with an extensive overlap of signal with RNApolIIser5 suggests a more general role of the protein in gene expression\textsuperscript{39,40}. The dASCIZ-HA-mCitrine reporter protein also displayed characteristic staining pattern on polytene chromosomes before and after heat shock treatment (induced by heating in a water bath at 37°C for 1 hr.). Successful induction of heat shock was verified by the redistribution of the RNApolIIser5 protein from numerous interbands on polytene chromosomes before treatment to the characteristic 87A/87C heat shock puffs after treatment (arrows in figure 4 G). Immediately after heat shock treatment, dASCIZ-HA-mCitrine’s signal from discrete staining was reduced or lost but that from global staining was retained (figure 4 E-H). The discrete staining was recovered after extended period of incubation at 25°C post treatment (1.5 hours to 5 hours after heat shock) (figure 4 I- P). Any changes in the protein level at the time-points examined before and after heat shock treatment is
being currently assessed. Hyperthermia induced redistribution has been reported for ATM-kinase interacting MRN complex proteins (Mre11, Rad51 and the NBS1) from the nucleus to the cytoplasm, upon heating of U-1 melanoma cells at 43.5°C for 1 hr. Such redistribution has been proposed to affect repair response upon genotoxic assault induced by IR and has been implicated in radiosensitization leading to cell death\textsuperscript{41}. In addition to fixed squash preparations, such dynamic distribution of dASCIZ-HA-mCitrine was also detected in salivary gland nuclei in live preparations (figure 4 Q-T). Taken together, these results show that dASCIZ-HA-mCitrine associates with the open regions of chromatin and displays a dynamic localization pattern on salivary gland polytene chromosomes.

\textit{dASCIZ is required for metamorphosis}

\textit{dASCIZ} mutants are pupal lethals. The exact time at which lethality occurs is not precise and varies from very early metamorphosis with the formation of brown puparium with no detectable differentiation of tissue, to late metamorphosis with dead pharate pupae. To further analyze the contribution of the protein in metamorphosis, homozygous mutants were selected as white prepupae as T=0, monitored through prepupal and pupal development and analyzed in accordance with Bainbridge and Bownes\textsuperscript{27}.

At 24 hours APF (after puparium formation), the WT population showed posteriorly displaced imago with proper imaginal disc eversion. Development of the adult cuticular structures like head, wings, legs and abdomen could be clearly
detected inside the pupal cuticle. The legs and wings were also fully extended along abdomen. The gas bubble could be clearly detected in the anterior end. Salivary glands could not be detected and green malphigian tubules were visible in the abdomen (figure 5 A). At this time-point, 25% of the mutant population was either decayed or undifferentiated without proper head or imaginal disc eversion (figure 5 C and D). In another 30% of the population, the gas bubble was still present posteriorly displacing the pupae anteriorly (image not shown). The remaining 45% of the mutant population that was not desiccated and had the correct gas bubble displacement displayed what we called the “Squishy” phenotype (figure 5 B). These pupae had little or no development of adult cuticular structures like head, wings, legs or abdomen inside the pupal cuticle. When poked with forceps, loose fat cells could be detected inside the pupal cuticle. In absence of the adult structures, the mutant imago lacked integrity or firmness and came to be called “Squishy”. (figure 5 B). An ecdysone pulse approximately at 10 hrs to 12 hrs post puparium formation, triggers programmed cell death of the larval salivary glands. To determine if this response is dependent on dASCIZ function, presence or absence of salivary glands was analyzed in mutant pupae at 24 h APF. Salivary glands were detected in 12 out of 13 mutant animals. In short, these observations indicate that the loss of dASCIZ affected early development during metamorphosis.

At 96 hours APF, the WT population showed bright red pigmentation in the eyes
with the full complement of bristles. The abdomen was segmented and developed, wings folded and almost black in color. Green meconium appeared dorsally at the posterior tip of the abdomen. Sex combs could be detected in males (figure: 5 E-H). At this time point, 52% of the mutant population appeared shriveled and decayed (figure 5 J). Another 30% of the population displayed the “squishy” phenotype and were almost indistinguishable from the 24-hours-APF mutants. It is possible that they stalled in development during early metamorphosis and made no further progress (figure 5 I). In the remaining 18% of the population, some rudimentary development of adult cuticular structures (head, wings, legs and abdomen) could be detected inside the pupal cuticle. Few bristles were present in the head and thorax. No pigmentation could be detected in the eyes (Figure 5 K).

Post 96 hours APF, while the WT population eclosed as adult flies, the \textit{dASCIZ} mutants did not register any event of eclosion. We kept documenting the fate of these mutants at 24-hour intervals. At 168 hours APF, 53% of the mutant population was shriveled, decayed and degenerated (figure 5 M). Another 33% of the population had the same “squishy” phenotype (figure 5 L). Interestingly, the remaining 13% showed further signs of development compared to the 96-hours-APF-mutants (figure 5 O-R). Further maturation of adult structures had taken place inside the pupal cuticle. Pigmentation in the eyes could be detected along with bristles in head, thorax, legs and abdomen. Tanning developed in some parts of the body including head and thorax. Mild to severe impairment in the
segmentation of the abdomen could be observed. Brown spots could be detected in the abdomen of some pupae of this fraction.

The different populations of mutant pupae described eventually degenerated and failed to eclose with no escapers. Taken together, these results suggest a critical role for dASCIZ-gene product during metamorphosis.

**Defective ecdysone signaling in the dASCIZ-mutants during early metamorphosis**

A major regulator of metamorphosis and subsequent eclosion is 20E triggered signaling cascade (commonly known as the ecdysone-signaling cascade) that is tightly controlled by the expression of 20E-regulated genes\(^23,42\). Defective 20E signaling caused by an aberrant expression of one or more genes of the signaling cascade has been implicated in impaired metamorphosis in previous reports\(^43\). Analysis of the phenotypes in the dASCIZ mutant pupae raised the possibility that these phenotypes were triggered by changes in expression of key 20E regulated genes. To test this hypothesis, we checked the expression of a set of 20E-regulated genes, namely, \(ECR\), \(E74\), \(BR-C\), \(\beta FTZ-F1\) (Competence factor), and \(E93\) at 3 time-points: at white puparium formation \((T_0)\), mid-prepupal period \((T_8\) measured as 8hrs after \(T_0)\) and early pupae after head eversion \((T_{14}\) measured as 14 hrs after \(T_0)\). The expression patterns of these 20E-regulated genes during early metamorphosis (from \(T_0\) to \(T_{14}\)) have been studied multiple times in previous
reports and used as a point of reference in this study\textsuperscript{44,45,46,47,48,49,43,50,51,52}.

RNA was isolated from white prepupae (T\textsubscript{0}), midprepupae (T\textsubscript{8}) and early pupae (T\textsubscript{14}) from both WT and mutant backgrounds, converted into cDNA and analyzed for the expression of aforementioned genes by RT-PCR\textsuperscript{53,54,51,55}. \textit{dASCIZ} expression was significantly reduced in the mutants at all time points checked (figure 6 F). The RT-PCR analysis showed that the expression of few ecdysone regulated genes was significantly affected at certain time-points (figure 6 A-E). Among them, the expressions of \textit{BR-C} and \textit{E93} were affected significantly at T\textsubscript{14} and T\textsubscript{8} respectively (figure 6 B and E). \textit{BR-C} is a key 20E inducible gene required for fruitfly metamorphosis and has been shown to perform a plethora of functions including fusion and morphogenesis of imaginal discs to form continuous and compact adult structures\textsuperscript{46,56,57}. Hence downregulation of \textit{BR-C} may account for the observed defects of the “squishy” mutant pupae. The expression of \textit{E93} was significantly decreased at T\textsubscript{8} but not at T\textsubscript{14}. Previous studies have shown that \textit{E93} is a key 20E inducible gene required for salivary gland histolysis in response to the 20E pulse at 12 hrs APF\textsuperscript{49,58}. The \textit{dASCIZ} mutants display persistent salivary glands yet do not suffer a significant \textit{E93} downregulation at T\textsubscript{14}. Preceding its expression in the salivary glands, \textit{E93} is induced in the larval midgut catalyzing the destruction of midgut gastric caeca and midgut shortening\textsuperscript{49}. A reduced expression of \textit{E93} in the \textit{dASCIZ} mid-prepupal mutant is likely to interfere with larval midgut destruction, generating yet another defect in metamorphosis\textsuperscript{49}. An
instance of E93 independent block in salivary gland cell death was reported in the Med24 (encodes for a component of the mediator complex) mutants\textsuperscript{59}. The Med24 mutants contained intact salivary glands at 14 hrs and 20 hrs APF but displayed no detectable aberration in the expression 20E regulated early genes including E93 and cell death regulators like rpr, hid. Apart from a severely reduced Med24 expression, the Med24 mutants also displayed a dramatic reduction in the Mdh2 (CG7998) expression\textsuperscript{59}. Expression of Mdh2 was significantly reduced in the dASCIZ mutant pupae at T\textsubscript{8} and T\textsubscript{14} (figure 6 G). We are currently analyzing Med24 expression in the dASCIZ mutant pupae at all time points. Altogether these results suggest that misregulation in expression of 20E regulated genes during early metamorphosis leads to defective metamorphosis in the dASCIZ mutant background.

**Disruption of dASCIZ impairs organ growth**

We next investigated if the dASCIZ-mediated impaired organ morphogenesis (little or no development of adult cuticular structures in the mutant squishy pupae) was penetrant in other developmental stages. We decided to examine the brain in the preceding stage of 3\textsuperscript{rd} instar larvae. Brains isolated from wandering mutant larvae also showed defective morphogenesis. Though these structures were present in the mutants without exception, their size was severely reduced (Figure 7.A and B). To understand if the observed difference in size was due to decreased cell proliferation or increased cell death or a combination of both, we performed the
following experiments:

- As a marker of DNA replication in S phase, we performed Bromodeoxyuridine or BrdU labeling in the brains (figure 7 C-F).

- As a marker of mitosis, we performed immunostaining with PH3 or phospho-Histone3 in the brains (Figure 7 G-J).

- To qualitatively evaluate the levels of apoptosis, we performed AO or Acridine orange staining in brains (figure 7 K-L).

In the brains of the 3rd instar larvae, DNA replication is primarily concentrated in 2 bands in each of the 2 optic lobes of the brain. These bands are referred to as the inner anlage and the outer anlage and are delineated by dense BrdU staining in bands (figure 7 C-D). In addition, some scattered neuroblasts in the central brain and ventral nerve cord are also reported to be sites of DNA replication (figure 7 C-D). These sites are represented by speckled BrdU staining outside the dense bands in the optic lobes. While some speckled BrdU staining could be detected in the scattered larval neuroblasts in the mutant brains, no staining in the outer or the inner anlage could be detected, revealing a severely reduced rate of DNA synthesis (figure 7 E-F). Next, we examined mitotic activity in the mutant brains. Mitotically active cells in Drosophila and other model organisms have been routinely analyzed by staining with antibodies against the M-phase marker PH3. As shown in figure, PH3 -positive foci were decreased in the mutant brains (figure 7 I-J) compared to the WT (figure 7 G-H).
To evaluate the contribution of cell death, we performed AO staining in the brains. AO staining has been shown to be highly selective for apoptotic cells. AO staining does not reveal any significant difference in apoptosis between the WT and the mutant (figure 7 K-L). In alignment with reports, some dead cells were detected in the WT brains.

Taken together, these observations suggest disruption of dASCIZ affects brain morphogenesis in mutant wandering 3rd instar larvae (another post-embryonic stage). Reduced DNA synthesis and mitotic activity were detected in the small mutant brains likely accounting for the defect.

In addition to metamorphosis, altered expression of the 20E response genes has been implicated in defective growth and proliferation, ultimately affecting the morphogenesis of organs in fruitfly. Currently, we are analyzing the expression of these response genes in the dASCIZ mutant brains. Spread of H3K9me2,3 has been shown to interfere with the transcriptional activation of 20E response genes causing developmental abnormalities. In addition, DNA hypermethylation accompanied by an increase in H3-K9 di- and tri-methylation has been shown to affect DNA synthesis and chromosome condensation in larval neuroblasts. In view of dASCIZ-HA-mCitrine’s predominant localization in the open regions of the chromatin, we are also evaluating H3K9me2,3 levels in mutant brains.

**Generation of the imprecise mutant IP3709**

The EP(3)3709 line was used as the template for generation of another mutant allele for dASCIZ by imprecise excision. The EP(3)3709 line containing an EP
element 27 base pairs downstream of the ATG initiating codon of \textit{dASCIZ} (CG14962) is shown in figure 8 A. Excision was achieved by crossing to the helper transposase line \(\Delta 2\)-3. The mini-\textit{white} \((w^+)\) associated with the EP element of \textit{EP(3)3709} was mobilized by the \(\Delta 2\)-3 transposase. A fly line in which the EP element sequence was excised was identified by its white-eye color and named \textit{IP3709} (IP for Imprecise excision). Individual males from that line were crossed to \(yw/yw; TM2/TM6b\) balancer females, and progeny were assessed for homozygous lethality. The imprecise excision was mapped using primers corresponding to sequences flanking the EP element insertion site by PCR or polymerase chain reaction. DNA isolation from single flies and PCR reactions were performed as described in Preston \textit{et al} in 1996\textsuperscript{33}. The forward primer and the reverse primer in the first set of primers used for the first sequencing reaction binds upstream of the EP insertion site and \(~216\) bps downstream of the ATG initiator codon respectively (figure 8 B). This primer set is designed to amplify a region of 216 bps from the WT background (figure 8 B). As shown in 8 C, amplicon size detected in the WT background is of appropriate size while that detected in the mutant background is higher as calibrated against the marker used. The next primer set binding to a region further downstream of the site of EP insertion is depicted in figure 8 D and used in the \(2^{nd}\) sequencing reaction. This set is expected to amplify a fragment of 401 bps in the WT background. As shown in figure 8 E, the \(2^{nd}\) PCR sequencing reaction detects a band of the right size in both the WT and the mutant backgrounds. Analysis of these 2 sequencing results
suggests that while the major part of the EP element has been excised, a region of ~300 bps has been retained in the IP3709/IP3709 background. This is because in the first PCR reaction, the higher band in the mutant migrates a little lower than the 500 bp band of the marker. Since the expected fragment size is 216 bps and the observed fragment size is ~450 bps (visual approximation), the additional 234 bps may have come from the EP element. That is to say, a part of ~234 bps from the EP element was probably left behind and the rest excised out due to imprecise excision. This preliminary PCR sequencing must be supplemented with multiple sequencing reactions using different primer sets to delineate the lesion in the IP3709 background. A cursory examination of the IP3709 homozygotes revealed they were pupal lethals with defective metamorphosis similar to homozygotes of the template line EP(3)3709 (data not shown). However, detailed analysis of dASCIZ expression as well as a rescue of lethality associated with the IP3709 homozygotes by transgene expression should be done to establish the IP3709 as a true mutant allele of dASCIZ.

**Discussion**

dASCIZ mutants analyzed in this body of work exhibit lethality in the pupal phase with no eclosion of a mature adult fly (figure 1 D). Inspection of the mutant pupae at different stages of development showed impaired metamorphosis with little or no morphogenesis of adult organs and appendages and interference with histolysis of larval tissues like the salivary glands (figure 5). Careful analysis of the
phenotypes in the mutant pupae suggested a defect in 20E responses during early metamorphosis similar to those reported for the mutants in the 20E signaling pathway\textsuperscript{43,66,67}. Indeed, expression of 20E-responsive genes, especially \textit{BR-C}, \textit{E93} and \textit{Mdh2} were down-regulated in the \textit{dASCIIZ} mutants during early metamorphosis (figure 6). The mutant brains as analyzed from the wandering 3rd instar larvae were also reduced in size than the WT controls (figure 7 A-B). Thus \textit{dASCIIZ} mutation mediated defective organ morphogenesis is not restricted to metamorphosis but is also penetrant in other post-embryonic developmental stages, the 3\textsuperscript{rd} instar larval stage in this case. Reduced BrdU and PH3 staining accompanied by no detectable increase in AO staining in the mutant brains likely account for the smallness of size (figure 7). Altered expression of 20E response genes in the 3\textsuperscript{rd} larval instar has been implicated in reduced growth of organs by impeding cell growth and proliferation\textsuperscript{64}. In view of that, we are currently analyzing the 20E response pathway by checking the expression of \textit{E74}, \textit{BR-C}, \textit{ECR}, \textit{E93} and \textit{βFTZ-F1} in age matched 3\textsuperscript{rd} instar larvae from both the WT and the \textit{dASCIIZ} mutant 3\textsuperscript{rd} instar larvae.

Though Atmin has been shown to be a nuclear protein, information on its chromatin binding properties is limited. In this study we have assessed the distribution of the dASCIIZ-fusion protein (dASCIIZ-HA-mCitrine) on giant salivary gland polytene chromosomes in relation to the active RNAPolIIser5. dASCIIZ-HA-mCitrine shows a wide-spread occupancy of the interbands on the
polytene chromosomes with an extensive overlap of signal with RNApolIser5. In associating with polytene chromosomes, it displays 2 types of staining patterns: global (the relatively low intensity signal detectable most of its sites of occupancy on polytene chromosomes) and discrete (the visibly elevated signal from few sites on polytene chromosomes) (figure 4 A-D). Proteins with such widespread distribution in euchromatin may play a general role transcription-related processes by creating and/or maintaining regions of open chromatin. If dASCIZ performs a similar role, then its disruption may effect a spread of the heterochromatin marker H3K9 di- or tri- methylation with the potential of impeding 20E response. An impaired 20E response was documented in the dASCIZ mutant pupae likely accounting for the defective metamorphosis. Experiments are underway to track their expression in the 3rd instar larvae as a possible trigger of defective organ morphogenesis. As a causal factor behind an aberrant 20E response, H3K9me2,3 levels are also being investigated in the mutant brains.

dASCIZ-HA-mCitrine displays characteristic distribution before and after heat shock on salivary gland polytene chromosomes (figure 4 E-P). Immediately after heat shock treatment, the dASCIZ-HA-mCitrine signal from discrete staining but not from global staining on polytene chromosomes becomes undetectable (figure 4 E-H). The signal from discrete staining becomes detectable after extended incubation at 25°C (figure 4 I-P and figure 5 Q-T). The functional significance of such behavior is not currently known and is a topic of future research. Overall,
study of the dASCIZ homozygous mutants during the 3rd instar larvae or metamorphosis suggests that the human ASCIZ or Atmin may play a role in post-embryonic stages of development like attainment of puberty (the human equivalent of fly metamorphosis) by regulating responses from sex-hormones. Studies on the chromatin-association properties of the transgenic fusion protein suggest a possible role of the protein in gene expression. One such role may be to mark and/or maintain open regions of chromatin. Experiments are underway to investigate the hypothesis and to understand its significance.

References


34. Note, A. Biotechnology Explorer Protein Electrophoresis of GFP:


Figures

(A)
Figure adapted from flybase.org
http://flybase.org/cgi-bin/gbrowse2/dmel/?Search=1;name=FBgn0035407
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Figure 1. Multiple sequence alignment of the human and fly proteins, genomic organization of CG14962 or dASCIZ and identification of an EP insertion mutant line EP(3)3709 as a putative dASCIZ mutant: (A) MUSCLE alignment of human Atmin (accession number: O43313) and Drosophila CG14962 or dASCIZ (accession number: NP_647776). Asterisk or * denotes full conservation between the residues compared. Colon or : denotes conservation between amino acids with strongly similar physico-chemical properties. Period or . denotes conservation between amino acids with weakly similar physico-chemical properties. (B) Genomic organization of CG14962 or dASCIZ. The solid yellow bar depicts CG14962 or dASCIZ gene. The solid light green bar depicts a portion of the CG32280 gene. The 2 genes are transcribed in opposite directions as shown by solid red and dark green arrows. The mRNA isoforms composed of exons (dark orange for dASCIZ and light orange for CG32280) and introns (thin straight lines...
in black) are shown beneath the gene representations. The light purple bar inside the largest dark orange bar stands for the coding sequence in both the \textit{dASCIZ} isoforms. The solid blue arrow points to the insertion of the EP element in the \textit{dASCIZ} coding sequence. (C) The EP element insertion in the \textit{EP(3)3709/EP(3)3709} background affects \textit{dASCIZ} as well as \textit{Cut-up} expressions. Transcripts of \textit{dASCIZ}, \textit{Cut-up} and \textit{CG32280} were examined by RT-qPCR from whole larval RNA extracts prepared from wandering 3\textsuperscript{rd} instar larvae. While the expressions of \textit{dASCIZ} (vertical light green bar) and its transcriptional target \textit{Cut-up} (vertical pink bar) were majorly affected, that of \textit{CG32280} (vertical yellow bar) suffered negligible decrease. Each bar depicts an average of 3 replicates. (D) Percentage eclosion of adult flies in different genetic backgrounds. 0\% eclosion was registered in the \textit{EP(3)3709/EP(3)3709} background compared to the 94\% in the WT background. Percentage eclosion was calculated as the number of empty pupal cases expressed as percentage of total pupae counted.
Figure 2. dASCIZ-HA-mCitrine expression in transgenic wandering 3rd instar larvae using different drivers. (A-C) Live expression of dASCIZ-HA-mCitrine fusion protein when driven by the Act5C-Gal4 driver. (A) Live dASCIZ-HA-mCitrine in salivary glands. (B) Live dASCIZ-HA-mCitrine in brains. (C) Live dASCIZ-HA-mCitrine in wing discs. (D-E) Expression of dASCIZ-HA-mCitrine when driven by the Sg3-Gal4 driver. (D) Live dASCIZ-HA-mCitrine in
salivary glands (E) Western blots of dASCIZ-HA-mCitrine probed with mouse anti-HA Ab and rabbit 1183 Ab. Protein extracts were prepared from salivary glands dissected from wandering 3\textsuperscript{rd} instar larvae from the transgenic, control and homozygous mutant backgrounds and immunoblotted with the aforewritten Abs. dASCIZ-HA-mCitrine detected by 1183 Ab (Left hand, upper panel) and anti-HA Ab (Right hand, upper panel) from salivary gland extracts from the transgenic background but not from the control or \textit{EP(3)3709/EP(3)3709} backgrounds. Tubulin was used as loading control (F) Amino acid sequence of dASCIZ showing the presence of 27 prolines (highlighted in yellow), 1 SQ (highlighted in turquoise blue) and 7 TQ (highlighted in light green).
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Figure 3. Act5C-Gal4 driven expression of dASCIZ-HA-mCitrine rescues the pupal lethality (scored as complete failure of eclosion of adult flies) of $EP(3)3709/EP(3)3709$: (A-B) Live expression of dASCIZ-HA-mCitrine detected in ovaries dissected from rescued females of genotype $dASCIZ-HA-mCitrinew+/Act5C-Gal4w+;EP(3)3709w+/EP(3)3709w+$. (A) Expression is detected in the FITC filter but not in the (B) TRITC filter underscoring the specificity of detection. (C) Chart showing the rescue of $EP(3)3709/EP(3)3709$ lethality by ectopically expressing dASCIZ-HA-mCitrine, scored as percentage eclosion of adult flies in experimental and control backgrounds. Transgene expression effects a 27% and 7% rescue of eclosion defect of the $EP(3)3709/EP(3)3709$ when driven (Act5C-Gal4 driver a) and non-driven respectively.
Figure 4. The transgenic reporter protein dASCIZ-HA-mCitrine associates with open regions of polytene chromosomes and displays a characteristic staining pattern before and after heat shock treatment: Squash preparations of polytene chromosomes triple labeled with anti-HA for dASCIZ-HA-mCitrine (green), anti-RNA polIIser5 (red) and Hoechst (Blue/gray). (A-D) dASCIZ-HA-
mCitrine shows staining of the open regions of the chromatin under “control” conditions (25°C) with extensive co-localization with the RNAPolIIser5 signal. (A) Composite (B) dASCIZ-HA-mCitrine stained with anti-HA. The fusion protein displays discrete staining (long arrow) and global staining (short arrow). (C) RNAPolIIser5 staining. (D) Hoechst staining. (E-H) Loss of dASCIZ-HA-mCitrine signal from discrete staining but not from global staining immediately after heat shock treatment. (E) Composite (F) dASCIZ-HA-mCitrine (G) RNAPolIIser5. Successful induction of heat shock was marked as the presence of 87A/C puffs is indicated by an arrow and stained by RNAPolIIser5. (H) Hoechst staining. (I-L) Slight but detectable recovery of discrete staining pattern (arrow) after 1.5 hrs of incubation at 25°C after heat shock treatment. (I) Composite (J) dASCIZ-HA-mCitrine. Arrow indicates re-appearance of discrete staining pattern. (K) RNAPolIIser5 staining (L) Hoechst staining. (M-P) Extensive recovery of discrete staining signal after 5 hrs of incubation at 25°C after heat shock treatment. (M) Composite (N) dASCIZ-HA-mCitrine. Arrows indicate the extensive re-appearance of discrete staining signal (O) RNAPolIIser5 (P) Hoechst staining. (Q-T) Live imaging of dynamic dASCIZ-HA-mCitrine signal before and after heat shock treatment. (Q) dASCIZ-HA-mCitrine signal before heat shock treatment (R) immediately after heat shock treatment (S) 1.5 hrs after heat shock and treatment (T) 5 hrs after heat shock treatment in salivary gland nucleus is shown in the lower panel. Arrows indicate elevated dASCIZ-HA-mCitrine signal at 25°C and its recovery after 1.5hrs and 5 hrs of incubation at 25°C after heat-shock treatment.
Figure 5. \textit{EP(3)3709/EP(3)3709} mutants are pupal lethals and fail to emerge
as adult flies. Defective development in the EP(3)3709/EP(3)3709 pupae staged at different time points during metamorphosis. For all the WT and most of the mutant pupae, the pupal shell was removed and the developing imago was analyzed microscopically. (A-D) Pupal development at 24 hrs APF: (A) WT imago. (B) “Squishy” mutant imago. (C) Desiccated mutant pupa with defective head eversion. (D) Undifferentiated mutant pupa. (E to K) Pupal development at 90 hrs APF: (E) Dorsal and (F) ventral WT male imago. Sex combs indicated by orange arrow in F. (G) Dorsal and (H) Ventral WT female imago. Green meconium indicated at the dorsal tip by green arrow in G. (I) Squishy mutant pupa (J) Desiccated mutant pupae. (K) A mutant pupa showing rudimentary development of adult cuticular structures inside the pupal cuticle and appearance of some bristles but no eye color. Post 90 hrs APF, the WT pupae eclosed as adult flies. No eclosion was recorded in the mutant background. (L-R) Mutant pupae at 168 hrs APF. (L) Squishy mutant imago (M) Desiccated mutant pupa. (O) Dorsal and (P) ventral image of a mutant male imago showing further development. (Q) Dorsal and (R) ventral images of a mutant female imago showing further development. The red arrow shows eye color, white arrow sex combs, blue arrow imperfect male genitalia, and purple arrow partial segmentation of abdomen. The thick red arrow shows eye color and green arrows the dark spots in the abdomen of mutant female imago.
Figure 6. Ecdysone response measured as a function of expression of ecdysone-regulated genes at $T_0$ (White prepupae) $T_8$ (8 hrs since $T_0$) and $T_{14}$ (14 hrs since $T_0$): Total RNA was isolated from whole wild-type and $EP(3)3709/EP(3)3709$ staged prepupae and pupae at designated time-points. 1 ug of RNA was converted into c-DNA and used in RT-PCR for assessing expression of ecdysone-regulated genes with $RP49$ as the internal control. Signal intensities
of RT-PCR products were measure by Image J software. Relative level of expression was determined by normalizing individual band intensity to RP49 band intensity. (A) Normalized expression of E74 at T₀, T₈ and T₁₄. (B) Normalized expression of BR-C at T₀, T₈ and T₁₄. (C) Normalized expression of ECR at T₀, T₈ and T₁₄. (D) Normalized expression of βFTZ-F1 at T₀, T₈ and T₁₄. (E) Normalized expression of E93 at T₀, T₈ and T₁₄. (F) Normalized expression of dASCIZ at T₀, T₈ and T₁₄. (G) Normalized expression of Mdh2 at T₀, T₈ and T₁₄. Each bar depicts the average of 3 replicates.
**Figure 7. Impaired morphogenesis in brains dissected from mutant wandering 3\textsuperscript{rd} instar larvae:** (A-B) Smaller brains in the mutant (A) WT brain (B) Mutant brain. (C-F) BrdU staining in WT and mutant brains (C) BrdU staining in WT brains. WT brains incorporated BrdU in the outer (Long arrow) and inner anlage (short arrow) located in the developing optic lobes. BrdU incorporation is pseudocolored in green. Speckled green spots in optic lobes indicate BrdU incorporation in some scattered neuroblasts. (D) As a counterstain, the DNA of the brain cells was marked with Hoechst and pseudocolored as gray. (E) BrdU staining in mutant brains. Mutant brains show some BrdU incorporation from scattered neuroblasts but little or no incorporation in the outer or inner anlage. (F) Hoechst staining of mutant brains pseudocolored in gray. (G-J) Anti-PH3 staining in WT and mutant brains. (G) Anti-PH3 staining in WT brains. WT brains showed increased PH3-positive foci. Anti-PH3 staining was pseudocolored in red. (H) As a counterstain, DNA of brains was marked with Hoechst and pseudocolored in gray. (I) Anti-PH3 staining in mutant brains. Reduced number of PH3 positive foci in the mutant brains (J) Hoechst staining of mutant brains. (K-L) AO staining in WT and mutant brains analyzed through the FITC filter. (K) AO staining in WT brains and (L) AO staining in mutant brains
Figure 8. Generation of imprecise excision mutant IP3709 and is preliminary characterization: (A) Insertion of the EP element in EP(3)3709. (B) Forward primer (Red) and reverse primer (blue) used in the 1st sequencing reaction. (C) Results of the first sequencing reaction is represented by the agarose gel image.
and described in the adjacent yellow box. (D) Forward primer (Red) and reverse primer (blue) used in the 2\textsuperscript{nd} sequencing reaction. (E) Results of the 2nd sequencing reaction is represented by the agarose gel image and described in the adjacent yellow box.
CHAPTER 4. FUTURE DIRECTIONS

Direction 1

Spread of heterochromatin in the \textit{dASCIZ}-mutant background as a probable causal factor behind downregulation of ecdysone response:

An inspection of the dASCIZ-HA-mCitrine distribution on the salivary gland polytene chromosomes reveals that the transgenic reporter protein associates with nearly every interband with an extensive overlap of signal with the transcriptionally active RNApolIIser5. Such distribution suggests that \textit{dASCIZ} may play a role in creating or recognizing and maintaining boundaries of open chromatin\textsuperscript{1,2,3,4}. In doing so, it may regulate transcription by facilitating the access of the transcription factors PolII to the open chromatin. Collapse of such boundaries and a subsequent spread of the silencing marker like the H3K9 di-, trimethylation has been implicated in a number of developmental defects\textsuperscript{5,6}. A report by Tsurumi \textit{et al.}, 2013 showed that loss of Kdm4 demethylases triggered a global increase of the heterochromatin marker tri-methyl H3K9, interfering with the proper expression of ecdysone response genes, ultimately leading to developmental arrest and death in the 2\textsuperscript{nd} larval instar\textsuperscript{6}. In addition, the pupal stage too, has been hypothesized to be particularly sensitive to hypermethylation\textsuperscript{5}. The \textit{dASCIZ} mutant pupae displayed defective metamorphosis with a mis-regulation of ecdysone response. Given the nature of dASCIZ-fusion protein’s chromatin labeling, a possible causal factor behind an impaired ecdysone response
could be the spread of the heterochromatin upon disruption of \(dASCIZ\). Hypermethylation in the \(dASCIZ\) mutant background can be first studied at the genomic level. Genomic DNA can be isolated from mutants at different stages of development and the level of cytosine methylation, as a mark of DNA hypermethylation, can be determined by capillary electrophoresis. In addition, the status of tri- and di- methyl H3K9, as a marker for constitutive and facultative heterochromatin, can be assessed in relation to Histone H3S10 phosphorylation and histone H4 acetylation by both immunostaining and western blotting. In the event of any alteration, either genomic or epigenetic or both, promoters of the ecdysone response genes especially \(BR-C\) can be inspected for an increase of the silencing mark by chromatin immunoprecipitation\(^6\). Studies in this direction will help us track the source of aberration in ecdysone response causing developmental defects.

**Direction 2**

**Misregulation of *Drosophila* fat body development and a corresponding energy deficit in \(dASCIZ\)-mutants:**

The fat body is essential for energy storage and utilization in fruitflies and is considered to be analogous to the vertebrate adipose tissue. In addition to being the central organ of energy storage, the insect fat body is an important site of essential biosynthetic and metabolic activity\(^7\)–\(^9\). In the life cycle of *Drosophila*
melanogaster, developmental stages are distinctly marked by feeding and non-feeding behaviors. For example, the larval instars till the early 3rd are dedicated to voracious feeding and are characterized by rapid growth in mass. In addition, the larva accumulates excess nutrients, primarily in the larval fat body. This is followed by the late 3rd instar stage when the larvae stops feeding and prepares for transition into pupae. The Drosophila pupal stage is a non-feeding stage and is characterized by extensive histolysis of most of the larval tissues. Interestingly, the larval fat is preserved but undergoes a distinct transformation from an organized tissue to a mass of very loosely associated fat cells. The growing imago inside the pupal case accesses the stored energy from these individual fat cells for their development and maturation. Abnormalities in the fat bodies have been linked to energy deficits and developmental defects, including pupal lethality with a failure to eclose\textsuperscript{10}. Mutant larvae from the dASCIZ background appeared more transparent than the WT counterparts. In addition, they displayed severely affected metamorphosis with a complete failure in eclosion. Most of the mutant pupae died during early metamorphosis with no detectable differentiation of organs and appendages. Some mutant pupae could make it to the later stages of development but died as dead pharate adults. Such impairment in the growth and maturation of organs and appendages like head, wings and legs coupled with 100% failure in eclosion suggest energy deficits and possible defects in fat body function. Increased transparency of the mutant larvae, as inspected in the 3rd larval instar, suggests a possible reduction in the amount of fat tissue. A population-based study
may be conducted at all the larval instars to deduce the penetrance and stage-specificity of this phenotype. To understand if this transparency is a result of defective fat bodies and reduced fat storage, fat bodies from both the mutants and the WT can be analyzed by a simple staining of lipophilic dyes like Nile Red. This can be followed by an evaluation of the overall TAG or triacylglycerol content from whole animals at different time points in development to assess the hypothesized energy deficit. In the event of any aberration in the fat body structure and/or TAG levels, the ultrastructure of the mutant fat bodies may be studied by TEM or transmission electron microscopy with a special attention to mitochondria. This is because, mitochondrial abnormalities will not only help explain the source of energy deficit, should there be any, it will also account for the reduced mdh2 expression seen in the mutant pupae\textsuperscript{11}.

In short, study of the larval fat body may help answer questions about energy deficits and defective developmental transitions. In addition, it may provide an insight into brain development of mutant larvae for it has been shown in a previous study that co-culture with fat body \textit{in vitro} induces quiescent imaginal neuroblasts, but not quiescent endoreplicating cells, to enter the cell cycle\textsuperscript{12}. 
References


9. Arrese, E. L. & Soulages, J. L. Insect fat body: energy, metabolism, and


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I want to thank my advisors Prof. Kristen Johansen and Jørgen Johansen for giving me an opportunity to pursue research under their supervision. Their constant encouragement and criticism during my doctoral study inspired me to do better science. Apart from teaching me the nuances of research, they taught me patience and perseverance during the rough years of my PhD. In that regard, my PhD would not have been possible without the help of Prof. Jack Girton. His guidance and support saw me through when I was struggling with complicated genetic crosses. The extensive discussions I had with him on various aspects of fly development helped me appreciate both the beauty and power of the fruitfly model.

I would like to thank my committee members Dr. Jeffrey Essner and Dr. Gustavo Macintosh for their helpful suggestions on my research and agreeing to serve in my Program of Study Committee.

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My parents constituted the main driving force behind my PhD. I would not have come this far without them and cannot thank them enough for all they have done for me. They taught me to see and seek positivity in everything, a lesson I
revisited every single day of my PhD. Another force in my life that held me from succumbing to the constant challenges and rigors of PhD is, Judhajeet, my better half. Throughout my PhD, he kept soaking me with the thought “Believe and act as if it were impossible to fail”. He empowers me everyday. Apart from my parents, I owe this degree to him.

I like to think that my life outside lab boosted my life inside lab. Books, music, films, food and few wonderful friends have made my stay in Ames, both fun and enriching.

And finally, this list would be incomplete without mentioning “Invictus” a poem written by an English poet, William Ernest Henley.

“\textit{I am the master of my fate,}
\textit{I am the captain of my soul.}”

These words formed the epicenter of my strength and revived my hopes whenever the going got tough.