Kinome-wide RNAi screen uncovers the role of *Drosophila* Ballchen in maintenance of epigenetic cell memory

by

Muhammad Haider Farooq Khan

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Advisor

Dr. Muhammad Tariq

Department of Biology

Syed Babar Ali School of Science and Engineering

Lahore University of Management Sciences

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Syed Babar Ali School of Science and Engineering

Lahore University of Management Sciences

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Muhammad Haider Farooq Khan

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TO MY TEACHERS

from whom I continue to learn

AND TO MY FAMILY MEMBERS

for their support, encouragement and love

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أعوذُ بِٱللَّهِ مِنَ ٱلشَّيۡطَٰنِ ٱلرَّجِيمِ

بِسمِ اللَّهِ الرَّحمٰنِ الرَّحيمِ

الحَمدُ لِلَّهِ رَبِّ العالَمينَ

الرَّحمٰنِ الرَّحيمِ

بِسمِ اللَّهِ الرَّحمٰنِ الرَّحيمِ

ما كانَ مُحَمَّدٌ أَبا أَحَدٍ مِن رِجالِكُم وَلٰكِن رَسولَ اللَّهِ وَخاتَمَ النَّبِيّينَ

اللّهُمّ صَلّ عَلَى مُحَمّدٍ وَآلِ مُحَمّدٍ

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June 10th 2020

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SYED BABAR ALI SCHOOL OF SCIENCE AND ENGINEERING

Dissertation Approval

The members of the Committee approve the dissertation entitled "Kinome-wide RNAi screen uncovers the role of Drosophila Ballchen in maintenance of epigenetic cell memory", defended on June 19, 2020.

It is recommended that this dissertation be used in partial fulfillment of the requirements for the degree of Doctor of Philosophy from Department of Biology in Syed Babar Ali School of Science and Engineering.

Dr. Muhammad Tariq
(Advisor)

Prof. Dr. Faiz ul Hassan Nasim
(External Examiner)

Prof. Dr. Irshad Hussain
(FDC Member)

Prof. Dr. Amir Faisal
(FDC Member)

Prof. Dr. Aziz Mithani
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Title

Kinome-wide RNAi screen uncovers the role of *Drosophila* Ballchen in maintenance of epigenetic cell memory

Submitted by: Muhammad Haider Farooq Khan

Advisor: Muhammad Tariq

Abstract

The capacity of cells to diversify in different cell lineages and the ability to remember their identity is central to the development of multicellular organisms. Once the cell fate is determined during early development, the identity of different cell types is maintained during subsequent development which involves maintenance of cell type specific gene expression patterns through successive cell divisions. Polycomb group (PcG) and trithorax group (trxG) proteins are evolutionarily conserved factors that maintain cellular identity after the establishment of cell fates. PcG proteins behave as repressors to maintain heritable patterns of gene silencing and trxG proteins act as anti-silencers by maintaining active gene expression profile linked to specific cell fate. Genetic and molecular analysis has revealed extensive details about how PcG and trxG antagonize to maintain cell fate, but the cellular signaling components that contribute to trxG mediated gene activation or PcG mediated repression have remained elusive. The aim of this thesis is to discover novel contributions of cellular signaling components, specifically protein kinases in facilitating trxG to counteract PcG mediated gene repression. To this end, an RNAi based reverse genetics approach is employed to determine the novel role of kinases and cell signaling proteins in maintenance of gene activation by trxG proteins in *Drosophila*. The *ex vivo* kinome-wide RNAi screen led to identification of twenty-eight genes shortlisted as potential regulators of trxG. Serine-threonine protein kinases from the primary list of candidates were validated by performing a secondary screen. *Drosophila* Ballchen (Ball), a histone kinase among the candidates, was further characterized as a novel trxG regulator. The *ball* (*ball*^{2}) mutant strongly suppressed the extra sex comb phenotype of *Pc* mutants and enhanced the loss of abdominal pigmentation phenotype of *trx* mutants. In addition, depletion of Ball in homozygous *ball*^{2} embryos and mitotic clones resulted in downregulation of trxG target genes. Interestingly, diminished amounts of H3K4 trimethylation and H3K27 acetylation, two histone marks associated with anti-silencing activity of trxG, were also observed in *ball*^{2} mitotic clones. Moreover, Ball co-localizes with Trx on chromatin and inhibits H2AK118 ubiquitination, which is a histone mark central to PcG mediated gene silencing. Together, this data suggests that Ball mediated phosphorylation

contributes to a binary switch at H2A which facilitates trxG to counteract repression by PcG. Thus, a novel role of a protein kinase, Ball, is discovered in controlling PcG/trxG mediated cell fate maintenance. Further molecular and biochemical characterization of hitherto unknown link between trxG and Ball will reveal effect of cell signaling in maintaining dynamic state of gene expression patterns involved in epigenetic cell memory.

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List of Abbreviations

Abbreviations	Words
5caC	5-carboxylcytosine
CpG	5'—C—phosphate—G—3'
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine
Ash1/2	Absent, small or homeotic disc1/2
Ash1L	Absent, small or homeotic disc 1 like
ATF2	Activating transcription factor 2
ATP	Adenosine triphosphate
AFM	Atomic force microscopy
AurB	Aurora kinase B
BMI1	B cell-specific Mo-MLV integration site1
Ball	Ballchen
BAF	Barrier to autointegration factor
Brm	Brahma
BAF	Brahma associated factor
BAF155/170/47	Brahma associated factor155/170/47
BAP55/180	Brahma associated protein55/180
BAP	Brahma associated proteins
BRG1	Brahma-related gene 1
BPTF	Bromodomain PHD Finger Transcription Factor
BRD4	Bromodomain-containing 4
CREB	cAMP response element binding protein
CK 1	Cell kinase 1
Caf1-55	Chromatin assembly factor1-p55 subunit
ChIP	Chromatin immuno precipitation
CBX2/4/6/ 7/8	Chromobox2/4/6/7/8
CHD1/7	Chromodomain helicase DNA-binding protein 1/7
CMT3	Chromomethylase 3
CDS	Coding sequence
cDNA	Complementary DNA
COMPASS	Complex of proteins associated with SET1
CBP	CREB binding protein
Cryo-EM	Cryo-electron microscopy
CTD	C-terminal domain
Cxxc1	Cxxc domain containing protein 1
CDK1	Cyclin-dependent kinase 1
CDK12	Cyclin-dependent kinase 12
CDK2	Cyclin-dependent kinase 2

Continued from previous page	
CKI	Cyclin-dependent kinase inhibitor
DAPI	4',6-Diamidino-2-Phenylindole
DNA	Deoxyribonucleic acid
DHSB	Developmental studies hybridoma bank
DOT1L	Disruptor of telomeric silencing 1-like
DNMT1	DNA methyltransferase1
dsRNA	Double stranded RNA
dRAF	dRING associated factor
dRING	Drosophila really interesting new gene
DRSC	Drosophila RNAi screening center
Dpy-30L1	Dumpy-30-like1
EED	Embryonic ectoderm development
ECL	Enhanced chemi-luminescence
EGFP	Enhanced green fluorescent protein
E(z)	Enhancer of zeste
EZH1/2	Enhancer of zeste homolog 1/2
EGF	Epidermal growth factor
EDTA	Ethylene-diamine-tetra-acetic acid
ESC	Extra sex comb
FXR	Farnesoid x receptor
Fs(1)h	Female, sterile, homeotic
FRET	Fluorescence resonance energy transfer
GFP	Green fluorescent protein
GEF	Guanine exchange factor
GTP	Guanine triphosphate
H1p	Phosphorylation of histone H1
HD2/3	Heidelberg2/3
HDAC6	Histone deacetylase6
H2AK118ub1	Histone H2A mono-ubiquitinated at lysine 118
H2AT119p	Histone H2A phosphorylated at threonine 119
H3K27ac	Histone H3 acetylated at lysine27
H3K27me2	Histone H3 di-methylated at lysine27
H3T3p	Histone H3 phosphorylated at threonine 3
H3K4me3	Histone H3 tri-methylated at lysine 4
H3K9me3	Histone H3 tri-methylated at lysine 9
H3K27me3	Histone H3 tri-methylated at lysine27
HRP	Horse radish peroxidase
Hcf	Host cell factor
HOTAIR	HOX antisense intergenic RNA
HOTAIRM1	HOX antisense intergenic RNA myeloid 1
HOTTIP	HOXA transcript at the distil tip

Continued from previous page	
IDG	Illuminating the druggable genome
IDE	Imaginal disc enhancer
JAK3	Janus kinase3
Jarid2	Jumonji AT-rich interactive domain 2
Jmj	Jumonji domain
JNK	Jun N-terminal kinases
kcnq1ot1	KCNQ1 opposite strand transcript 1
Kis	Kismet
KYP	Kryptonite
L	Liter
LSD1	Lysine specific demethylase1
KDM2A/B	Lysine specific demethylase2
KMT	Lysine specific methyl transferase
KDM2A	Lysine-specific demethylase2A
MET1	Maintenance methyltransferase 1
MEL18	Melanoma nuclear protein18
Mnn1	Menin1
MeCP2	Methyl-CpG binding protein 2
µl	Microliter
mA	Milli-ampere
mg	Milligram
ml	Milliliter
MSK	Mitogen-and Stress-activated protein Kinases.
MLL1/2/3/4/5	Mixed-lineage leukemia 1/2/3/4/5
Mor	Moira
mESCs	Mouse embryonic stem cells
MEN1	Multiple endocrine neoplasia1
ng	Nano-gram
NIH	National institute of health
ncRNA	Non-coding RNA
NMR	Nuclear magnetic resonance
Ncoa6	Nuclear receptor coactivator 6
NHK-1	Nucleosomal histone kinase 1
NuA4	Nucleosome acetyltransferase of H4
PBS	Phosphate-buffered saline
PIP4,5	Phosphoinositol-4,5-bisphosphate
PEM	PIPES, EGTA $MgCl_2$ Buffer
Plk3	Polo-like kinase 3
PBAF	Polybromo containing brahma associated factors
PBAP	Polybromo containing brahma associated protein complex
Pc	Polycomb

Continued from previous page	
PcG	Polycomb group
PRC1	Polycomb repressive complex1
PRC2	Polycomb repressive complex2
Ph	Polyhomeotic
ph-p	Polyhomeotic proximal
PCR	Polymerase chain reaction
PBX	Postbithorax
Psc	Posterior sex comb
PTMs	Post-translational modifications
Pa1	PTIP associated 1
Ring1A/B	Really interesting new gene 1A/B
RGC	Receptor Guanylate Cyclases
RBAP46/ 48	Retinoblastoma associated protein 46/48
Rbbp5	Retinoblastoma-binding protein5
RNA	Ribonucleic acid
RYBP	Ring and YYI protein
RNAi	RNA interference
rpm	Rotation/minute
Sce	Sex combs extra
Sox2	Sex determining region y-box2
STAT	Signal transducer and activator of transcription
siRNA	Small interfering Ribonucleic Acid
SAXS	Small-angle X-ray scattering
Snr1	Snf5-related1
SDS	Sodium dodecyl sulfate
SAGA	Spt-Ada-Gcn5 acetyltransferase
SAM	Sterile-a-motif
SETD2	Su(var), enhancer of zeste, trithorax (SET) domain containing 2
SET1	Su(var)3-9, E(z), trx 1 [SET] protein 1
Sayp	Supporter of activation of yellow protein
SUV39H2	Suppressor of variegation 3-9 homolog 2
Su(z)12	Suppressor of zeste 12
Su(z)2	Suppressor of zeste 2
SWI/SNF	Switch/sucrose non-fermentable
TAF3	TATA-Box Binding Protein Associated Factor 3
Tet1/2	Ten-eleven translocation methylcytosine dioxygenase 1/2
TET	Ten-eleven translocation
TFIID	Transcription Factor II D
TE	Tris-EDTA
trx	trithorax
trxG	trithorax group

Continued from previous page	
Trr	trithorax related
Tb	Tubby
TK	Tyrosine kinases
TKL	Tyrosine kinases like
Utx	Ubiquitously transcribed x chromosome tetratricopeptide repeat protein
VRK	Vaccinia related kinase
VRK1	Vaccinia-related kinase 1
Wdr5	Wd repeat domain 5
Wdr82	Wd repeat domain 82
WARD	Wds, Ash2, Rbbp5, Dpy30-L1
Wds	Will die slowly
XIST	X-inactive specific transcript

Publications

1. Zain Umer*, Jawad Akhtar*, **Muhammad Haider Farooq Khan***, Najma Shaheen, Muhammad Abdul Haseeb, Khalida Mazhar, Aziz Mithani, Saima Anwar, and Muhammad Tariq. Genome-wide RNAi screen in *Drosophila* reveals Enok as a novel trithorax group regulator. Epigenetics and Chromatin 12, 55 (2019). https://doi.org/10.1186/s13072-019-0301-x

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1 INTRODUCTION

In metazoans, each cell type has its own specific identity that enables it to perform a particular function. This specific identity of a cell is rooted in its transcriptional memory, which is established earlier in development by a process called cell fate determination. Different cells attain diverse fates by transcriptional activation of altered combinations of developmentally important genes. The particular combination of active genes that a cell lineage expresses needs to be maintained and inherited mitotically for its faithful adherence to the already established fate. Different cell types utilize various epigenetic mechanisms in a combinatorial manner to make sure the established fate is maintained throughout life, such mechanisms include DNA methylation (Holliday and Pugh, 1975), histone modifications (Allfrey et al., 1964), non-coding RNAs (Jones et al., 1999; Matzke et al., 2001; Volpe et al., 2002), nucleosome remodeling (Narlikar et al., 2002; Sudarsanam and Winston, 2000) and chromatin compaction (Heitz, 1928). The evolutionarily conserved trans-acting factors that control the maintenance of established cell fate include two groups of proteins; Polycomb group (PcG) and the trithorax group (trxG). Through post-translational modifications of histones and regulation of nucleosomal structure, the PcG and the trxG proteins stabilize heritable repression and activation of gene expression, respectively. This process of cell fate maintenance mediated by PcG and trxG is conserved throughout metazoans (Brand et al., 2019; Cavalli and Heard, 2019).

Polycomb Repressive Complex 1 and 2 (PRC1 and PRC2) are the two major multi-protein complexes that belong to PcG. These complexes mono-ubiquitinate histone H2A at lysine 118 (H2AK118ub1) and di- or tri-methylate histone H3 at lysine 27 (H3K27me2, H3K27me3) respectively, whereas, trxG is quite heterogeneous because activation of transcription is a complex process with several steps involved. Many trxG proteins are also involved in general transcription along with specifically counteracting the PcG mediated repression (Kassis et al., 2017; Schuettengruber et al., 2017).

Initially, trxG proteins were discovered in *Drosophila* as positive regulators of developmental genes, thus antagonizing the PcG mediated gene repression

(Kennison and Tamkun, 1988). Although trxG is associated with active transcriptional state of genes and PcG is linked to gene repression, it remains an enigma why both groups occupy their target genes regardless of their expression state (Beisel et al., 2007; Papp and Müller, 2006; Ringrose et al., 2004). Since proteins of both groups also co-occupy the same loci on chromatin (Orlando et al., 1998; Strutt and Paro, 1997), this adds to the complexity of the question how the decision between gene activation or repression is made. Extensive work has been carried out to understand how PcG mediates gene repression and how trxG antagonizes PcG, however, it is not clear what decides which of the two prevails over the other. In this context, it is plausible to assume that cell signaling pathways can be of prime importance due to their ability to respond to intra and extracellular changes as well as their capacity to influence nuclear factors involved in gene repression or activation of specific genes. Cell signaling components, especially the protein kinases, regulate a repertoire of cellular processes by modifying more than two-third of cellular proteins' structure and function (Ardito et al., 2017), but interestingly to date both PcG and trxG lack kinases, apart from Fs(1)h. In *Drosophila,* Fs(1)h is the only kinase present in canonical trxG members but this too is an atypical kinase with no known kinase domain (Chang et al., 2007) which performs its recognized functions via its bromodomain (Kockmann et al., 2013). Although different processes linked to epigenetic inheritance, such as maintenance of chromosomal architecture to cell division, are regulated by protein kinases, cell signaling for the maintenance of gene activation by trxG or repression by PcG remains elusive. The question of how cell signaling pathways contribute to the epigenetic inheritance as well as maintenance of gene expression states linked to epigenetic cell memory during development remained unanswered.

To identify protein kinases that contribute to the maintenance of gene activation by trxG, an RNA interference (RNAi) based reverse genetics approach was employed during this dissertation because this approach is extremely efficient to screen large number of genes simultaneuously in a cell based assay. Moreover, consequence of RNAi mediated knockdown leads to partial loss of function instead of a complete knock out (Heigwer et al., 2018). An *ex vivo* kinome-wide RNAi screen was carried

out for this purpose, using a previously characterized reporter in *Drosophila* cell culture (Umer et al., 2019). In this reporter, the *Firefly* luciferase gene is expressed under the *Ubx* promoter and minimal *bxd* PRE. Minimal *bxd-PRE* was previously characterized to maintain the functionality of full-length *bxd* (Fritsch et al., 1999). Besides, *PBX (postbithorax)* (Zhang et al., 1991) and *IDE (Imaginal Disc Enhancer)* (Christen and Bienz, 1994) enhancers are also in the reporter to enhance its robustness, thus named *PBX-bxd-IDE-F.Luc* (*PRE-F.Luc*). The sensitivity of the *PRE-F.Luc* is also well characterized as it responds to varying concentrations of PcG in a dose-dependent manner. The reporter also efficiently responds specifically to the knockdown of different trxG members (Umer et al., 2019). The primary RNAi screen resulted in the identification of 28 cell signaling proteins that impaired maintenance of reporter expression. The list of identified genes generated from the kinome-wide RNAi screen is heterogeneous, just as the trxG itself is diverse. The majority of the candidates were protein kinases, but regulatory subunits of kinase complexes, kinase inhibitors, nucleotide kinases and a few lipid kinases, were included in the list. Remarkably, the only trxG member with predicted kinase activity; Fs(1)h was also in the list, confirming the functionality of the screen. Some other direct interactors of PcG/trxG proteins like CDK1 were also present in the list. CDK1 is known to control global epigenetic landscape in ESCs as well as to phosphorylate a plethora of epigenetic regulators (Michowski et al., 2020). A subset of these candidates, which mainly comprises serine-threonine kinases, was selected to validate the effect on the *PRE-F.Luc* reporter gene by performing a secondary screen. We have generated data suggesting protein kinases' role in regulating the PcG/trxG system, opening new avenues to understand how trxG may maintain active gene expression.

Moreover, this dissertation describes detailed genetic and molecular analysis of Ballchen (Ball), a histone protein kinase and a candidate gene from the kinome-wide screen that may regulate maintenance of gene activation together with trxG. Importantly, Ball is a known nucleosomal histone kinase that phosphorylates histone H2A at threonine 119 (H2AT119) (Aihara et al., 2004). I have characterized genetic interaction of *ballchen* (*ball*) mutant with PcG/trxG system and demonstrated that Ball exhibit trxG like behavior by strongly suppressing extra sex comb phenotype in

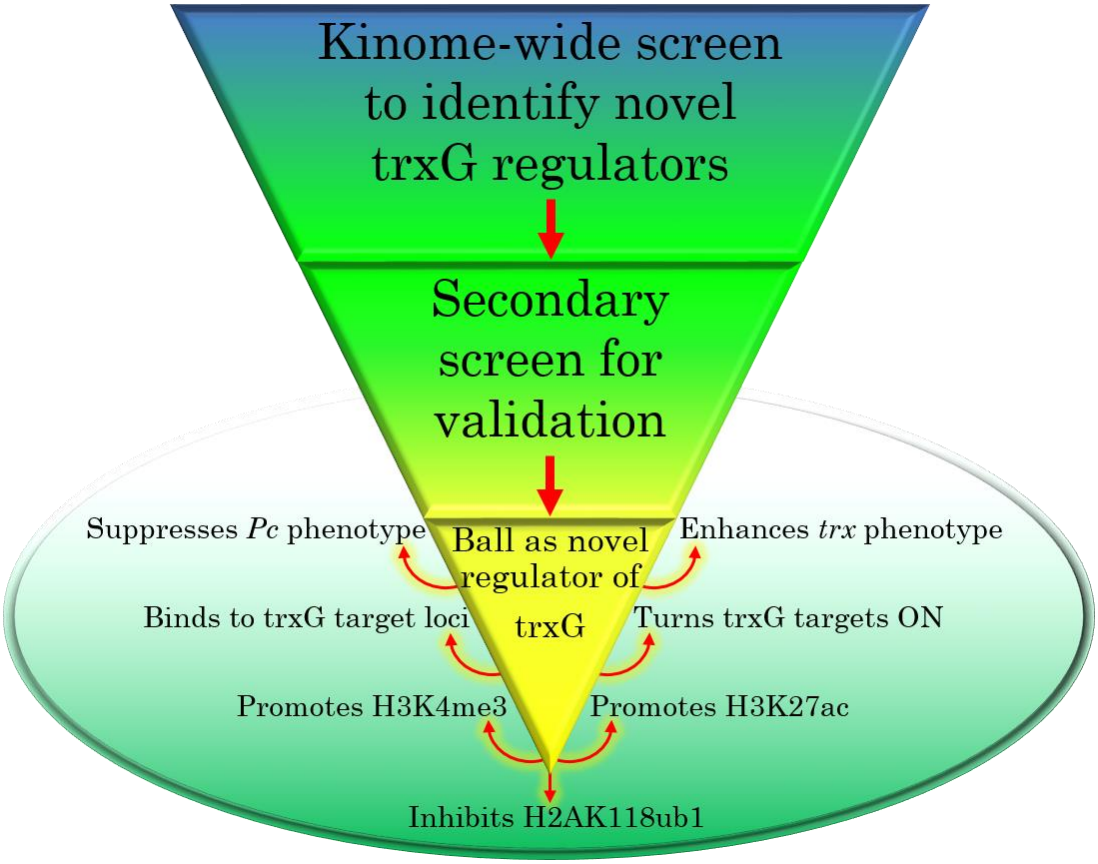

Figure 1.1: Framework of this dissertation. An *ex vivo* kinome-wide RNAi screen was executed to investigate signaling proteins that regulate trxG mediated gene activation. Twenty-eight genes were shortlisted as potential regulators of trxG mediated transcription activation. Serine-threonine protein kinases from the list were further validated by performing a secondary screen. A histone kinase, Ball, was further characterized as a novel trxG regulator. Mutant of *ball* (*ball*^{2}) strongly suppressed the extra sex comb phenotype of *Pc* mutants and enhanced the loss of abdominal pigmentation phenotype of *trx* mutants. Ball depletion in homozygous *ball*^{2} embryos and mitotic clones resulted in the downregulation of trxG target genes. Depletion of H3K4 trimethylation and H3K27 acetylation, two histone marks associated with anti-silencing activity of trxG, was also observed in *ball*^{2} mitotic clones. Moreover, Ball co-localizes with Trx on chromatin and inhibits H2AK118 ubiquitination, a histone mark central to PcG mediated gene silencing.

Pc mutants. It was also validated by strong enhancement of *trx* homeotic phenotype by *ball*. Importantly, expression of homeotic and non-homeotic targets of trxG was reduced due to depletion of Ball in mutant flies for *ball* and in cells treated with *ball* RNAi. Moreover, Ball also co-localizes with Trx at chromatin and inhibits PRC1 mediated mono-ubiquitination of histone H2A at lysine 118 (H2AK118ub1), thus counteracting PcG mediated repression. The findings acquired in this dissertation are summarized in **Figure 1.1**.

1.1 Aims and objectives

Although, some of the PcG and trxG proteins are known to be phosphorylated and phosphorylation of certain histone marks are also known to affect epigenetic cell memory, genetic and molecular evidence about the role of specific kinases or cell signaling proteins in epigenetic cell memory have remained elusive. The specific aim of this study was to discover the novel contribution of kinases and cell signaling proteins involved in maintenance of gene activation by trxG. In light of the above mentioned questions, specific aims and objectives of my doctoral thesis are as following.

In particular, this dissertation is aimed at:

- To uncover a link between novel or known cell signaling kinases with epigenetic cell memory governed by trxG.
- To establish genetic and molecular link between Ball and PcG/trxG system.

The specific aims of my dissertation were achieved by addressing following objectives:

- Execution of a kinome-wide RNAi screen for identifying kinases linked to the trxG mediated maintenance of epigenetic cell memory.
- Analysis of list of candidates from the RNAi screen.
- Validation of selected candidates by performing a secondary screen.
- Genetic and molecular analysis of *Drosophila* Ball with respect to PcG/trxG system.
- Establishment of a molecular link between Ball and trxG in maintenance of gene activation.

2 LITERATURE REVIEW

2.1 Epigenetic memory

The term epigenetics was first coined by Waddington in 1942 (Waddington 1942), who defined it as "the study of the processes by which the genotype brings the phenotype into being" (Waddington, 1942). Over the years, epigenetics was redefined by various renowned scientists (Holliday, 1994; Lappalainen and Greally, 2017; Nanney, 1958; Nicoglou and Merlin, 2017; Riggs et al., 1996) with the most recent definition as "the study of molecules and mechanisms that can perpetuate alternative gene activity states in the context of the same DNA sequence" (Cavalli and Heard, 2019). The mechanisms that perpetuate alternative gene activity states are either stable such as in genomic imprinting and X chromosome inactivation or dynamic such as histone modifications and inheritance of gene activity state during cell cycle. The epigenetic mechanisms that have intrinsic plasticity, essential for the maintenance of cell identities and for appropriately responding to the environmental signaling, are often described as epigenetic memory mechanisms (D'Urso and Brickner, 2014; Steffen and Ringrose, 2014). In this section, different epigenetic memory mechanisms will be discussed.

2.1.1 Regulation of epigenetic memory

Epigenetic memory is regulated in at least three different layers; a transcriptional layer of epigenetic memory, cellular layer, and transgenerational layer. Transcriptional memory is that a cell establishes a particular state of gene activity in response to some external stimuli and memorizes the state during its existence (D'Urso and Brickner, 2014), while cellular memory is when the memorized gene activity states are due to earlier developmental signals and are kept intact during cell cycle. Transgenerational memory is the inheritance of gene expression patterns across two or more generations (Cavalli and Heard, 2019).

2.1.2 Transcriptional memory

For the survival of a multicellular organism, each cell must adapt, improvise and respond to the external environmental changes. Such responses are due to the changes in the expression levels of several genes simultaneously. Following the

change in gene expression patterns, some of the responding genes are kept poised for faster reactivation. This phenomenon of rapid activation of previously expressed genes is called transcriptional memory. For example, in yeast exposed to salt stress, the genes for responding to oxidative stress are kept poised for quick response in case of future increase in salt levels (Guan et al., 2012). Cells use epigenetic transcriptional memory to deal with the natural stresses, extracellular signaling cascades and changes in environmental conditions.

2.1.3 Cellular memory

In metazoans, each cell has its own specific identity that specializes the cell to perform a particular function. This specific identity of a cell is rooted in its cellular memory that is established earlier in development by a process named cell fate determination. The cell fate is maintained during subsequent cell divisions by several cellular components including Polycomb and trithorax groups of proteins (see **Section 2.3** and **2.4**), DNA methylation (Holliday and Pugh, 1975; Riggs, 1975), noncoding RNA (Brockdorff et al., 1992; Brown et al., 1992; Lee et al., 1993; Wightman et al., 1993), the 3D architecture of genome (Lieberman-Aiden et al., 2009; Loubiere et al., 2019) and mitotic bookmarking of histone modifications or transcription factors (John and Workman, 1998; Martínez-Balbás et al., 1995; Michelotti et al., 1997). Such cellular components that contribute to heritable patterns of gene expression constitute the epigenome of a cell (Cavalli and Heard, 2019) and provide epigenetic barriers that prevent alterations in cell identity and gene expression patterns across multiple cell cycles (Brand et al., 2019).

2.1.4 Transgenerational memory

In normal physiological conditions, germline reprogramming resets the epigenome to accomplish successful reproduction. Still, the expression pattern of several loci in both animals and plants have been shown to inherit epigenetic memory of previous generations (Heard and Martienssen, 2014; Perez and Lehner, 2019). For example, in *Drosophila*, heat shock and osmotic stress are known to induce phosphorylation of a transcription factor ATF2 that is known to co-localize with HP1 on chromatin. This phosphorylation disrupts the heterochromatin formation and the defective chromatin state is transgenerationally heritable if heat shock is given to embryos

over multiple generations (Seong et al., 2011). Transgenerational epigenetic inheritance is not limited to lower animals but is also an established phenomenon in mammals. One such example is of agouti mice in which the expression state of a gene *Avy* is altered if mothers feed on diet supplemented with methyl donors resulting in inheritance of expression state in future generations. The altered expression pattern is due to altered DNA methylation patterns (Cropley et al., 2006). This transgenerational epigenetic memory not only helps organisms to effectively adapt to the environment but at the same time can negatively affect the survival of the offspring.

2.2 Gatekeepers of epigenetic cell memory

2.2.1 DNA methylation

Modifications in the DNA bases were known even in the middle of the previous century (Hotchkiss, 1948) but the association of such modifications with gene regulation was first proposed in 1975 by Holliday and Pugh (Holliday and Pugh, 1975). Once CpG islands were discovered (Bird et al., 1985) and DNA methylation was linked with gene repression (Razin and Riggs, 1980), the next big step towards understanding DNA methylation functionality was the cloning and characterization of DNA methyltransferase *DNMT1* gene that is responsible for 5- methylcytosine (5mC) maintenance (Bestor and Ingram, 1983; Gruenbaum et al., 1982) and MeCP2, the first protein known to recognize methylated DNA (Meehan et al., 1992, 1989). As described earlier, epigenetic memory has the property of intrinsic plasticity but such plasticity was questionable without any known DNA demethylase. A breakthrough came in this regard when 5- hydroxymethylcytosine (5hmC) was identified (Kriaucionis and Heintz, 2009). Soon after this discovery, an enzyme class TET (ten-eleven translocation) was identified that convert 5mC to 5hmC while generating chemical intermediates like 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) (Ito et al., 2010; Tahiliani et al., 2009). Thymine-DNA glycosylase in mouse embryonic stem cells can remove 5caC that eventually forms an active DNA demethylation pathway (He et al., 2011). Although DNA methylation is the first epigenetic mechanism that was discovered but it is not the mechanism that is fundamental to epigenetic memory in many organisms.

2.2.2 Histone modifications

The fundamental unit of chromatin is a nucleosome that is made up of a histone octamer consisting of two units of each of the histones H2A, H2B, H3 and H4 (Kornberg, 1974; Luger et al., 1997). All of the histones undergo post-translational modifications, also referred to as histone marks that include acetylation, methylation, phosphorylation and ubiquitination and others on several amino acid residues. Most of such modifications are on unstructured N- terminal or C- terminal tails of the histones that protrude outwards from the nucleosome. These histone tails likely enhance access of proteins to nucleosomal DNA and also facilitate cross-talk between histone marks and their chromatin binding factors (see **Table 2.1** also **Section 2.4** and **2.5.2.2**). Enzymes that catalyze specific modifications on histone tails are termed as writers, enzymes that reverse a specific modification are referred to as erasers and proteins that recognize a specific modification and contributes to materializing molecular consequence of a modification are called readers (**Table 2.1**) (Baek, 2011; Banerjee and Chakravarti, 2011; Gong et al., 2016; Hyun et al., 2017; Lawrence et al., 2016; Rossetto et al., 2012; Wang and Higgins, 2013; Zhao and Shilatifard, 2019). The fundamental roles of histone modifications in epigenetic memory mechanisms can be highlighted by the fact that some of the modifications and their subsequent effects are even more conserved across organisms than DNA methylation, for example, histone H3K9 methylation and its recognition by HP1 for gene repression (Allshire and Ekwall, 2015; Elgin and Reuter, 2013; Pikaard and Scheid, 2014).

Table 2.1: A subset of histone modifications along with their writers, erasers, readers and specific molecular functions.

Modifications	Writers	Erasers	Readers	Function
Methylation				
H3K4me1/2/3	COMPASS, COMPASS- like	KDM1A/B, KDM2A/B, KDM5A/B/C/D	TFIID, BRWD2/PHIP, MLL, TAF3, CHD1, RAG2, BPTF, PHF2/6/8, KDM4A	Transcriptional activation
H3K9me1/2/3	Suv39H1/2, G9a, GLP, SETDB1	KDM1A, KDM3A/B, JMJD1C, KMD4A/B/C/D	HP1, ATRX	mono-: transcriptional activation, di-, tri-: transcriptional repression, X-inactivation, imprinting
H3K27me1/2/3	EZH1/2	KDM6A/B, KDM7A, PHF8	EED, PC, CBX7	Transcriptional repression
H3K36me1/2/3	ASH1L, NSD1–3, SETD2/3, SETMAR, SMYD2	KDM2A/B, KDM4A/B/C/D, JHDM1A	ZYMND11, PHF19, LEDGF	Transcriptional elongation, repression, DNA repair
H3K79me1/2/3	DOT1L	-	-	Transcriptional activation
H4K20me1	PR-Set7	LSD1n	CRB2, p53BP1	Transcriptional activation
H4K20me2/3	SUV4-20H1/2	LSD1n, DPY-21	CRB2, p53BP1, JMJD2	Transcriptional silencing
Acetylation				
H3K4ac	GCN5, p300	Hst1, Sir2, HDAC3	BAF180	Transcriptional activation
H3K9ac	PCAF/GCN5	HDACs, SIRT1/6	BRD4/7, BRDT, BAF180, PCAF, BRM, BRG1, TAF1, MOZ, MORF, AF9, ENL, GAS41	Transcriptional activation
H3K14ac	CBP/p300, PCAF/GCN, TIP60	HDACs, SIRT1	ATAD2, BAZ2B, BRD2/3/4/7, BRDT, BAF180, PCAF, BRM, BRG1, TAF1, MOZ, MORF, DPF3	Transcriptional activation
H3K23ac	MOZ/MOF	HDACs	BRDT, BAF180, TRIM24, TRIM33	Transcriptional activation

Continued from previous page				
H3K27ac	CBP/p300	HDACs	AF9, ENL, GAS41	Transcriptional activation
H4K5ac	HAT1, CBP/p300, TIP60	HDACs	BPTF, BRD2/3/4/7, BRDT, BRM, TAF1, ZMYND8	DNA repair, transcriptional activation
H4K16ac	MOF	HDACs, SIRT1/2/3	BPTF, BRD2/3/4/7, BRDT, BRM, BRG1, TAF1, ZMYND8, TRIM24	DNA repair, transcriptional activation
Phosphorylation				
H2AT120p (H2AT119p)	Bub1, VRK1, Aurora B	Unknown	Shugoshin, Fe65	Cell division
H2AXS139p	ATM, ATR, DNA-PK, RSK2, MSK1	PP2A, Wip1, PP6 and PP4	MCPH1, MDC1	DNA repair, apoptosis
H3T3p	Haspin, VRK1	Repo-man/PP1	Survivin	Cell division
H3T6p	PKCβ, PKCα			Maintain gene activation
H3S10p	Aurora B, MSK1/2, PIM1, VRK1, JNK	Repo-man/PP1, PP2A	14-3-3, GCN5	Cell division, transcriptional activation
H3T11p	Mek1, CHK1, PKN1, PKM2	Repo-man/PP1	WDR5, GCN5	DNA repair, transcriptional activation
H3S28p	Aurora B, MSK1/2	Repo-man/PP1	14-3-3	Cell division, transcriptional activation
H3Y41p	JAK2	-	HP1	Differentiation, transcriptional activation
Ubiquitination				
H2AK119ub	Ring1A/B	BAP1, USP16, USP21, 2A-DUB, USP3, USP22	-	Transcriptional repression
H2BK120ub	RNF20, RNF40	USP3, USP7, USP22	-	DNA repair, transcriptional activation

2.2.3 Noncoding RNA

Even though the involvement of non-coding RNA molecules in epigenetic memory is a much recently discovered phenomenon than DNA methylation and histone modifications, its significance cannot be neglected. Many non-coding RNAs are

involved in transcriptional regulation but specific non-coding RNAs are also known to regulate transcription. For example, a 2.2kb long *HOTAIR (HOX antisense intergenic RNA)* (Rinn et al., 2007) acts as scaffold for LSD1 demethylase and PRC2 complex to couple H3K27 methylation with H3K4 demethylation and mediate transcriptional repression of *HOXD* genes cluster (Tsai et al., 2010). Contrarily, a 3.7kb long *HOTTIP* RNA *(HOXA transcript at the distal tip)* is known to directly recruit methyltransferase complexes that mediate H3K4 trimethylation and activate transcription of *HOXA* genes cluster (Wang et al., 2011). Different variants of another RNA called *HOTAIRM1* preferentially bind to either UTX/MLL or PRC2 complexes to mediate activating as well as silencing marks (Wang and Dostie, 2017). Many reports advocate that it is non-coding RNAs that regulate both DNA methylation (Jones et al., 1999; Kawasaki and Taira, 2004; Matzke et al., 2001) as well as histone modifications (Gu et al., 2012; Liu et al., 2007b; Volpe et al., 2002).

2.2.4 Interactions between DNA methylation, histone modifications and ncRNAs

The interplay between DNA methylation, histone modifications and ncRNAs suggests that the three interact with each other to provide feedback (Tariq and Paszkowski, 2004). For example, SUVH2 and SUVH9, two direct regulators of the siRNA pathway bind to methylated DNA and contribute to further DNA methylation (Johnson et al., 2014). Another example of such positive feedback loop is a cross-talk proposed between MET1 (maintenance methyltransferase1), CMT3 (Lindroth et al., 2001), and an H3K9 methyltransferase, KYP in *Arabidopsis* (Jackson et al., 2002; Tariq et al., 2003). CMT3 recognizes methylated H3K9 via its chromo and BAH domains (Du et al., 2012) and on the other hand, KYP binds to methylated DNA via its SRA domain (Johnson et al., 2007). Thus, methylated DNA recruits KYP to further methylate H3K9 and methylated H3K9 recruits CMT3 to further methylate DNA (Bartee et al., 2001; Du et al., 2012; Jackson et al., 2002, 2004; Lindroth et al., 2001) creating a strong positive feedback loop. Interestingly, RNA directed DNA methylation is also promoted by H3K9 methylation as siRNA pathway associated protein SHH1 has been shown to recognize methylated H3K9 (Law et al., 2013).

DNA methylation, histone modifications and the ncRNA pathways all converge on the PcG/trxG mediated cell memory. The examples of *HOTAIR* and *HOTTIP* already described in the previous section respectively recruit PcG and trxG proteins to their target genes. PcG proteins are also recruited to inactivated X-chromosome by *XIST* and *REPA* long ncRNA (Zhao et al., 2008). Some other long ncRNAs including *Kcnq1ot1* (KCNQ1 opposite strand transcript 1) are also known to recruit PcG proteins to the chromatin (Pandey et al., 2008). Similarly, the dependence of H3K27 methylation, a histone modification that is a hallmark of PcG mediated gene repression, on DNA methylation connect PcG with DNA methylation. The same study reported loss of Polycomb Repressive Complex 2 from its target genes' promoters and subsequent loss of PcG mediated gene repression in DNA hypomethylated cells (Reddington et al., 2013). As for the involvement of histone modifications in PcG/trxG mediated memory, many proteins of both groups are themselves histone modifiers, readers or erasers, details of which are given in subsequent sections.

2.3 Polycomb and trithorax groups of proteins

PcG and trxG proteins are molecular stamps that the cell machinery uses to inscribe the heritable state of epigenetic memory on chromatin. The two sets of proteins work antagonistically to each other in the cellular context. PcG inscribe the repressed state of its target genes while trxG antagonize it by working as anti-silencer for the same targets. Both PcG and trxG are linked to their specific chromatin modifications hence, such modifications play a crucial role in the maintenance of cell type-specific gene expression patterns (Turner, 2002). The conservation of PcG and trxG throughout metazoans highlights their significance in the maintenance of epigenetic memory (Loubiere et al., 2019). In this section, the core complexes of PcG and trxG with their respective functions will be described.

2.3.1 Polycomb group proteins

In multicellular organisms, developmental genes are highly regulated and are turned off in cells that do not require their function (Lewis, 1978). To make sure that such genes remain suppressed outside of their spatio-temporal requirement, PcG proteins are employed by the cell. PcG proteins ensure repression of key developmental genes which must be maintained in a silent state for inheritance of specific cell fates

determined during early development (Lewis, 1978; Struhl and Akam, 1985). Molecular function and mechanism of PcG mediated repression involve histone methylation, ubiquitination, protein-protein interactions and chromatin binding. Polycomb repressive complex 1 and 2 (PRC1, PRC2) are the two main multiprotein complexes, constituting the core members of PcG, that mediate PcG mediated gene silencing (Cavalli and Heard, 2019; Marasca et al., 2018).

2.3.1.1 Polycomb repressive complex 1

Polycomb (Pc), Posterior sex combs (Psc), Sex combs extra (Sce) and Polyhomeotic (Ph) are the four core components of PRC1 (Francis et al., 2001). Suppressor of zeste 2 [Su(z)2] can replace Psc as both are functional homologs (Lo et al., 2009). Each of the core proteins of PRC1 has its characteristic domains which are summarized in **Table 2.2**. Pc protein contains chromodomain (Paro and Hogness, 1991) which recognizes methylated lysine in histone tail (Cao et al., 2002; Czermin et al., 2002). Chromodomain of Pc specifically recognizes and binds with tri-methylation of H3K27 residue, thus mediate PRC2 dependent recruitment of PRC1 (Cao et al., 2002; Czermin et al., 2002; Müller et al., 2002). Sce/dRING has E3 ubiquitin ligase activity which catalyzes the ubiquitination of lysine 118 of histone H2A (Wang et al., 2004). dRING forms the core of PRC1 together with either Psc or Su(z)2 by interacting through RING finger domains. Psc and Su(z)2 have homology region that is essential for their incorporation into PRC1 that also contains the RING finger domain (Brunk et al., 1991; van Lohuizen et al., 1991). Two adjacent genes called *polyhomeotic-distal* and *polyhomeotic-proximal* code for the paralogous Ph proteins. The Sterile-α-motif (SAM), present in Ph proteins is shown to be essential for PRC1 mediated gene silencing (Gambetta and Müller, 2014). A related complex of PRC1 called dRING associated factors (dRAF) complex, that does not contain Polycomb has also been immunoprecipitated in *Drosophila*, which leads to demethylation of H3K36me2 (Lagarou et al., 2008). H3K36me2 is a histone mark catalyzed by a trxG member, Ash1 (An et al., 2011; Tanaka et al., 2007; Yuan et al., 2011). Thus by demethylation, the dRAF complex inhibits trxG mediated gene activation (Lagarou et al., 2008). The core members of the dRAF complex are Psc, Sce and a demethylase KDM2 (Lagarou et al., 2008). RYBP is another component of PRC1 that is known to mediate PRC2

independent recruitment of PRC1 in mESCs (Tavares et al., 2012). Although RYBP of *Drosophila* origin is not characterized to mediate PRC2 independent recruitment of PRC1 but its mutant flies were shown to be rescued by mammalian homolog, suggesting its similar function in *Drosophila* (Basu et al., 2014). Nonetheless, it has been reported that RYBP and another protein called YAF2 of mammalian PRC1 directly recognize the histone H2A ubiquitination mark and recruit PRC1 that catalyze further ubiquitination of neighboring nucleosomes through a positive-feedback loop mechanism (Arrigoni et al., 2006; Zhao et al., 2020).

2.3.1.2 Polycomb repressive complex 2

The tri-methylation of histone H3 at lysine 27 (H3K27me3) is the hallmark of PcG mediated gene silencing which is written by PRC2 (Cao et al., 2002; Czermin et al., 2002; Müller et al., 2002). PRC2 has four core components namely Enhancer of zeste [E(z)], Extra sex combs (Esc), Suppressor of zeste 12 [Su(z)12] and Chromatin assembly factor 1-p55 subunit (Caf1-55) (Müller et al., 2002; Ng et al., 2000; Tie et al., 2001). Each of the core proteins of PRC2 has its characteristic domains which are summarized in **Table 2.3**.

Table 2.2: PRC1 proteins along with their human orthologs, protein domains and known epigenetic roles.

Name	Human Orthologs	Notable domains	Epigenetic role
Sce (Sex combs extra)	RING1A/B (Really interesting new gene)	RING finger domain	H2AK118 ubiquitination, protein-protein interaction
Psc (Posterior sex combs)	BMI1 (B cell-specific Mo-MLV integration site 1)	RING finger domain	H2AK118 ubiquitination, protein-protein interaction
Su(z)2 (Suppressor of zeste 2)	MEL18 (melanoma nuclear protein 18)	RING finger domain	A functional homolog of Psc
Pc (Polycomb)	CBX2/4/6/7/8 (Chromobox)	Chromodomain	H3K27 tri-methylation binding
Ph-p (Polyhomeotic proximal), Ph-d (polyhomeotic distal)	PHC1/2/3 (Polyhomeotic)	Sterile Alpha Motif (SAM)	Oligomerization, looping of chromatin
Rybp (Ring and YY1 binding protein)	RYBP (Ring and YY1 binding protein)	Zinc finger domain	DNA binding activity
KDM2 (Lysine specific demethylase 2)	KDM2B (Lysine specific demethylase 2B)	JmjC, CxxC	H3K36 demethylase, DNA binding

Table 2.3: PRC2 proteins along with their human orthologs, protein domains and known epigenetic roles.

Name	Human Orthologs	Notable domains	Epigenetic role
E(z) (Enhancer of zeste)	EZH1/2 (Enhancer of zeste)	SET	H3K27 methyltransferase
Su(z)12 (Suppressor of zeste 12)	SUZ12 (Suppressor of zeste 12)	Zinc finger, VEFS	Possible DNA/RNA binding, Protein-protein interaction
Esc (Extra sexcombs)	EED (Embryonic ectoderm development)	WD40	H3K27 mono-, di-, tri-methylation binding
Caf1-55/Nurf55 (Chromatin assembly factor 1, p55 subunit)	RBAP46/48 (Retinoblastoma associated protein)	WD40	H3K36 trimethylation binding
Jarid2 (Jumonji, AT-rich interactive domain 2)	JARID2 (Jumonji, AT-rich interactive domain 2)	Zinc finger, ARID	H2AK118 ubiquitination binding, DNA binding

E(z) is the enzymatic subunit of the complex causing H3K27 trimethylation (H3K27me3) through its SET domain. Without methyltransferase activity of E(z), genes repressed by PcG are reactivated (Cao et al., 2002). Both Esc and Su(z)12, but not Caf1-55, are shown to be essential for the enzymatic activity of E(z) (Ketel et al., 2005; Nekrasov et al., 2005; Ohno et al., 2008; Wang et al., 2006). Known function of Esc in the PRC2 complex is of a scaffold protein that interacts with other proteins via its WD40 repeat domains. Seven WD40 repeats of Esc makes a β-propeller structure that facilitates protein-protein interactions (Ng et al., 1997; Tie et al., 1998; Wang et al., 2006). As mentioned earlier, Su(z)12 is also essential for H3K27me3 as a nonsense mutation in Su(z)12 has been shown to severely affect H3K27me3 (Lee et al., 2015). It has been proposed that the only cellular function both Esc and Su(z)12 is their involvement in PcG mediated gene silencing because both proteins have not been found in any other multi-protein complex. Phenotypes associated with mutants of both these genes are also consistent with such a hypothesis (Kassis et al., 2017).

Unlike Esc and Su(z)12, Caf1-55 is present in other multiprotein complexes (Suganuma et al., 2008) and it is not required for the enzymatic activity of PRC2 (Ketel et al., 2005; Nekrasov et al., 2005). Although the Caf1-55 role in PRC2 mediated gene silencing is unclear so far, it shares the structural features of seven

WD40 repeat domain constructing β-propeller (Song et al., 2008). In 2012, all four core components of PRC2 were co-purified with FLAG epitope-tagged Jarid2 (Herz et al., 2012a). Recently, Jarid2 is reported to recognize H2A ubiquitination via its ubiquitin interacting motif (Cooper et al., 2016; Kalb et al., 2014).

2.3.2 Trithorax group proteins

trxG is a heterogeneous group of proteins that activate transcription by mainly modifying histone tails and ATP dependent chromatin remodeling (Piunti and Shilatifard, 2016). One cellular function that unifies this diverse group of proteins is their role in counteracting PcG mediated gene silencing of the developmental genes. As compared to PcG proteins, trxG is less characterized and these proteins are also involved in various cellular processes apart from maintaining cell memory. Such cellular processes include general transcription, cell proliferation (Kingston and Tamkun, 2014), stem cell self-renewal (Ang et al., 2011), regeneration and tumorigenesis (Bagchi et al., 2007), apoptosis (Tyagi and Herr, 2009), and cell cycle (Liu et al., 2007a). The epigenetic roles of selected trxG proteins are given in **Table 2.4**, **Table 2.5** and **Table 2.6**.

2.3.2.1 COMPASS and COMPASS-like complexes

The complex of proteins associated with SET1 (COMPASS) is a histone methyltransferase complex that is known to methylate histone H3 lysine 4 (H3K4) residue (Miller et al., 2001; Roguev et al., 2001). There are three COMPASS/COMPASS-like complexes with each containing unique methyltransferase enzymes which are, SET1, Trithorax (Trx) and Trithorax related (Trr). SET1 complex (COMPASS) is the main source for di- and tri-methylation of H3K4 (Ardehali et al., 2011; Hallson et al., 2012), while Trx is associated with mono- and di-methylation of H3K4 at only enhancers and PREs (Rickels et al., 2016; Tie et al., 2014). On the other hand, Trr is shown to mono-methylate H3K4 only on enhancers specifically (Herz et al., 2012b). Apart from the core enzymes, four proteins are essential for each COMPASS complex that are termed as WARD (Wds, Ash2, Rbbp5, Dpy30-L1). SET1 complex additionally contains Cxxc1, Hcf1 and Wdr82 while COMPASS- like complex having Trx, additionally contains Hcf1 and Menin1 protein (Piunti and Shilatifard, 2016). COMPASS-like complex with Trr also contains another enzymatic subunit Utx

that is known to demethylate H3K27, the hallmark histone modification of PcG mediated gene silencing. Other unique subunits of the Trr-Utx complex include Ncoa6 and Pa1 (Schuettengruber et al., 2017).

Table 2.4: Proteins from COMPASS and COMPASS-like Complexes along with their human orthologs, protein domains and known epigenetic roles.

Name	Human Orthologs	Notable domains	Epigenetic role
Set1 (Su[var]3-9, Enhancer of Zeste, Trithorax 1)	SET1A/B (SET domain containing 1A/B)	SET	H3K4 mono-, di-, tri-methyl transferase
Trx (Trithorax)	MLL1/2 (KMT2A/B) (Mixed-lineage leukemia 1/2)	SET	H3K4 mono-, di-, tri-methyl transferase
Trr (Trithorax-related)	MLL3/4 (KMT2C/D) (Mixed-lineage leukemia 3/4)	SET	H3K4 mono- methyl transferase
Wds (Will die slowly)	WDR5 (WD repeat domain5)	WD40 repeat	Histone binding
Ash2 (Absent, small or homeotic disc 2)	ASH2L (Absent, small or homeotic disc-like)	zinc finger	DNA binding
Rbbp5 (Retinoblastoma-binding protein 5)	RBBP5 (Retinoblastoma-binding protein 5)	WD40 repeat	Histone binding
Dpy-30L1 (Dumpy-30-like 1)	DPY30 (Dumpy-30)	-	-
Wdr82 (WD repeat domain 82)	WDR82 (WD repeat domain 82)	WD40 repeat	Histone binding
Mnn1 (Menin 1)	MEN1 (multiple endocrine neoplasia 1)	-	-
Cxxc1(CXXC domain containing protein 1)	CFP1	CXXC domain	DNA binding
Hcf (Host cell factor)	HCF1 (Host cell factor 1)	Kelch	-
Pa1 (PTIP-associated 1)	PA1 (PTIP-associated 1)	-	-
Utx (Ubiquitously transcribed X chromosome tetratricopeptide repeat protein)	UTX (Ubiquitously transcribed X chromosome tetratricopeptide repeat protein)	JmjC	H3K27 demethylase
Ncoa6 (Nuclear receptor coactivator 6)	NCOA6 (Nuclear receptor coactivator 6)	-	-

2.3.2.2 SWI/SNF complexes

The most extensively studied chromatin remodeling complexes that are part of trxG are BAP (Brahma associated proteins) and PBAP (Polybromo containing BAP) (Kingston and Tamkun, 2014). Brahma protein is the ATPase subunit of both the complexes. Both the complexes are highly conserved from yeast to humans and have seven common subunits in *Drosophila*, including Brahma (Mohrmann et al., 2004). BAP complex is more closely related to SWI/SNF of yeast and BAP complex of humans, while PBAP is more closely related to the RSC complex of yeast and PBAF complex of humans. Moira is one of the seven common subunits of both complexes that is a homolog of the SWI3 subunit of the SWI/SNF complex (Arnoud J et al., 2000; Crosby et al., 1999). It forms the functional core of both complexes, together with Brahma (Phelan et al., 1999). The other five common subunits include BAP60, BAP111, BAP55, Snr1 and Actin (Schuettengruber et al., 2011).

In addition to seven common subunits, the BAP complex contains Osa and PBAP complex contain Polybromo, BAP170 and Sayp proteins that distinguish both complexes from each other (Chalkley et al., 2008; Collins, 1999). Osa is related to Swi1 of the SWI/SNF complex in terms of homology and contains an ARID domain for the facilitation of DNA binding of the BAP complex (Collins, 1999). No PBAP specific subunits were found in earlier genetic screens that identified trxG genes that speculated PBAP involvement in general transcription, but suppression of Pc mutant phenotypes by Sayp mutation confirms PBAP involvement in trxG mediated anti-silencing functions (Kassis et al., 2017).

2.3.2.3 Other complexes of trxG

The founding member of trxG, the Trx protein is found to be present in a complex, other than COMPASS-like, known as the TAC1 complex. TAC1 complex contains CREB (cAMP response element) binding protein (CBP), that is responsible for acetylation of histone H3K27 (Tie et al., 2014). The existence of the TAC1 complex is somewhat controversial due to three reasons. Firstly, it has not been reconstituted *in vitro* by recombinant proteins, secondly, it is immunoprecipitated from embryos but not present in S2 cells of embryonic origin (Mohan et al. 2011) and thirdly, any human homolog of such complex is yet to be identified (Kassis et al., 2017). Another

Table 2.5: Proteins from SWI/SNF Complexes along with their human orthologs, protein domains and known epigenetic roles.

Name	Human Orthologs	Notable domains	Epigenetic role
Brm (Brahma)	BRM/BRG1 (Brahma/ Brahma related gene 1)	helicase, bromodomain	ATPase-dependent chromatin remodeling
Osa	BAF250A/B (Brahma associated factor 250A/B)	ARID	possible DNA binding
Mor (moira)	BAF155/170 (Brahma associated factor 155/170	SWIRM, SANT, chromodomain	possible DNA and histone binding
Snr1 (Snf5-related 1)	BAF47 (Brahma associated factor 47)	winged helix	possible DNA binding
Sayp (Supporter of activation of yellow protein)	BAF45A-D (Brahma associated factor 250A/B45A-D)	PHD-finger	possible DNA binding
Bap55 (Brahma associated protein 55)	BAF53A/B (Brahma associated factor 53A/B)	actin-like	-
Bap180 (Brahma associated protein 180)	BAF180/BAF200 (Brahma associated factor 180/200)	polybromodomain	histone binding
(Brahma associated protein 55)	BAF60A-C (Brahma associated factor 60A-C)	Swi-B	-
Bap111 (Brahma associated protein 111)	BAF57 (Brahma associated factor 57)	HMG	possible DNA binding
Actin5C	beta-ACTIN	-	-

Table 2.6: Miscellaneous trxG proteins that do not belong to COMPASS or SWI/SNF complexes, with their human orthologs, protein domains and known epigenetic roles.

Name	Human Orthologs	Notable domains	Epigenetic role
Ash1 (Absent, small or homeotic disc 1)	ASH1L (Absent, small or homeotic disc 1 like)	bromodomain, SET	H3K36 methyltransferase
CBP (CREB binding protein)	CBP (CREB binding protein)	bromodomain, HAT	H3K27 acetyltransferase
Fs(1)h (Female, sterile, homeotic)	BRD4 (bromodomain-containing 4)	bromodomain, NET	Histone binding, protein-protein interaction
Kis (Kismet)	CHD7 (chromodomain helicase DNA-binding protein 7)	ATPase, chromodomain	Chromatin remodeling

important complex of trxG proteins is the Ash1 complex that was earlier associated with histone H3K4 methylation (Nastase Byrd and Shearn, 2003) but now known to only di-methylate histone H3K36 (Tanaka et al., 2007; Yuan et al., 2011). Ash1 is known to physically interact with a bromodomain-containing trxG protein Fs(1)h encoded by *fs(1)h* gene (Kockmann et al., 2013). The established function of Fs(1)h protein is to act as a reader of acetylated histone and form protein-protein interactions (Kockmann et al., 2013) but its human homolog, BRD4, is also shown to act as an atypical kinase without an identifiable kinase domain. BRD4 phosphorylates RNA Pol-II at the C-terminal domain and subsequently promotes transcription (Devaiah et al., 2012). It is speculated that Fs(1)h may also perform a similar function in *Drosophila* (Chang et al., 2007). Apart from Brahma, *Drosophila* also contains another important ATP dependent chromatin remodeler called Kismet (Kis) in the context of trxG mediated gene activation. Kismet protein contains ATPase domain as well as two chromodomains (Daubresse et al., 1999) that hint towards its direct binding to methylated histone proteins. It has been shown that both Trithorax and Ash1 enrichment on chromatin is decreased in the absence of Kis (Dorighi and Tamkun, 2013) that further stress upon its epigenetic role.

2.4 The interplay of PcG and trxG complexes

PcG and trxG proteins form several multiprotein complexes that primarily read (recognize), write (catalyze), or erase (remove) different histone modifications, as explained in the previous section. In this section, the interactions of different PcG complexes, interactions of different trxG complexes, and how both groups antagonize each other's functions will be described, especially in the context of histone modifications, and consequent state of gene expression.

2.4.1 Interactions between PcG complexes

Both the PRC1 and PRC2 complexes not only reinforce their function by recognizing their own marks but also positively influence each other to perform their repressive function. The PRC2 is responsible for H3K27 methylation that is read by both PRC1 via Pc (Cao et al., 2002; Czermin et al., 2002) and by PRC2 via EED (Margueron et al., 2009). Similarly, the catalytic subunit of PRC1, Sce/dRING is responsible for the H2AK118ub mark (H2AK119ub in mammals) (Wang et al., 2004) that is recognized by

PRC1 via RYBP and YAF2 (Arrigoni et al., 2006; Zhao et al., 2020) and by PRC2 via JARID2 (Cooper et al., 2016; Kalb et al., 2014). Interestingly, two of the PcG complexes also exhibit reciprocal molecular functions, which is ubiquitination of H2A by PRC1 (Wang et al., 2004) and deubiquitination by PR-DUB complex of the same residue (Scheuermann et al., 2010). PR-DUB may also have other targets apart from H2A that may explain its role in PcG mediated gene silencing.

2.4.2 Interactions between trxG complexes

On the flip side trxG proteins also reinforce their gene activation function by catalyzing histone modification and then reading the same marks to further establish the active transcription state of the target genes. For example, the H3K4 methylation mark, catalyzed by COMPASS complexes, is also read by the PHD domain containing proteins including PCF1 (Eberl et al., 2013; Murton et al., 2010; Shi et al., 2007) and MLL1 of the same complexes (Wang et al., 2010b). Nucleosomal remodeling complex NURF, an ISWI remodeling complex, is also known to interact with H3K4 methylation via its subunit BPTF (Wysocka et al., 2006). In yeast, the SAGA complex is also known to bind H3K4me2 and H3K4me3 via its sgf29 subunit that utilizes its tudor domain to recognize these histone marks (Bian et al., 2011). Another histone acetyltransferase NuA3 complex also recognizes H3K4 methylation by the PHD domain of its subunit Yng1 to efficiently acetylate H3K14 (Taverna et al., 2006). Interestingly, the SAGA and NuA4 (homolog of mammalian TIP60) histone acetyltransferase complex is known to increase the retention of SWI/SNF complexes on promoter regions (Hassan et al., 2001). For more efficient transcription initiation, TFIID also binds to H3K4 methylation by its PHD domain containing TAF3 subunit (Lauberth et al., 2013; Vermeulen et al., 2007). Just like PR-DUB and PRC1, trxG also has a demethylase, Lid, that erases the COMPASS mediated H3K4 methylation mark (Eissenberg et al., 2007; Lee et al., 2007; Secombe et al., 2007). **Figure 2.1** summarizes how PcG complexes reinforce each other and how trxG works synergistically to antagonize PcG mediated gene repression.

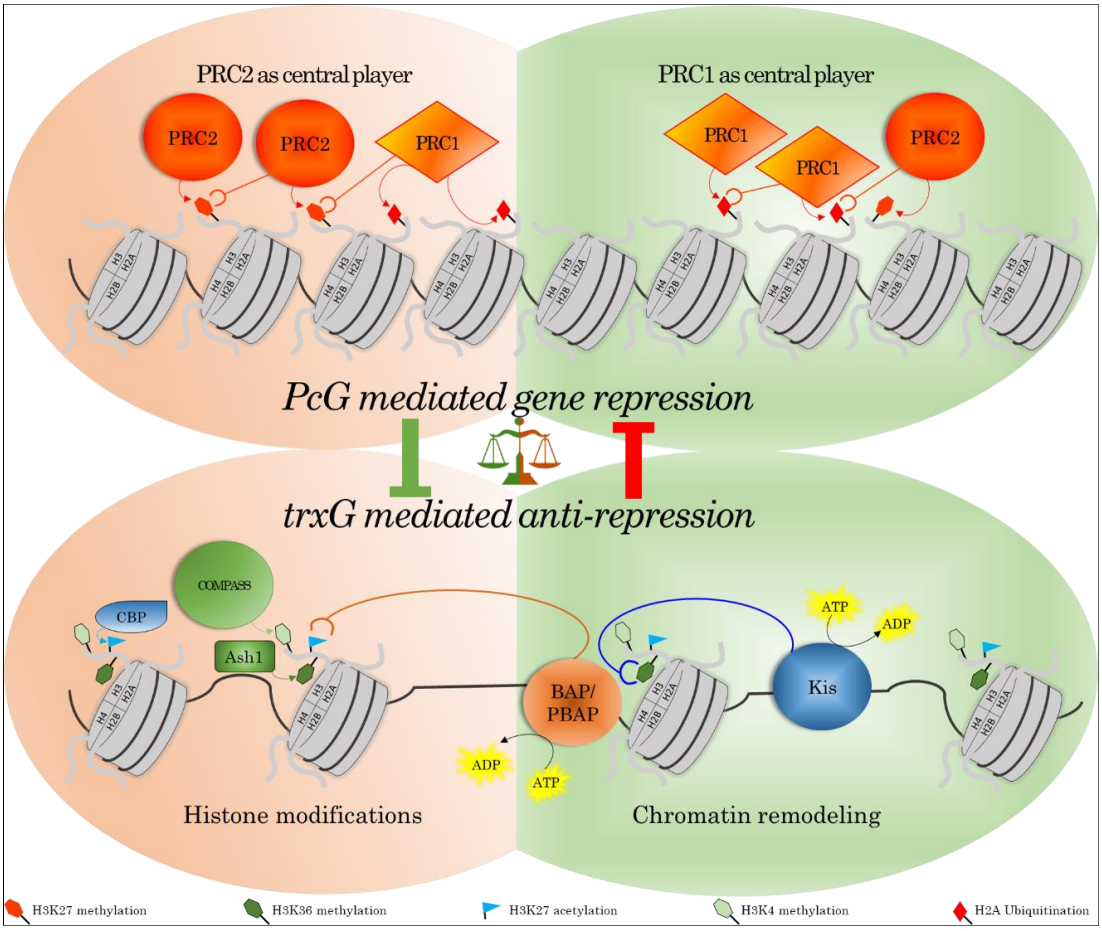

Figure 2.1: Crosstalk between PcG and trxG protein complexes. PRC1 and PRC2, the two core complexes of PcG reinforce each other by recognizing histone marks deposited by these complexes. PRC1 recognizes PRC2 mediated H3K27 methylation through Pc, and PRC2 recognizes PRC1 mediated H2A ubiquitination through Jarid2. Both complexes also reinforce their own binding to chromatin by recognizing their own marks too. PRC1 recognizes H2A ubiquitination through RYBP and YAF2, while PRC2 recognizes H3K27me through EED. Such reinforcements lead to cycles of histone modifications and their recognition, which further modify histones and recruit more PcG proteins to mediate gene repression. trxG proteins antagonize PcG by placing their own histone modifications and by remodeling of chromatin. The COMPASS complexes mediate H3K4 methylation, Ash1 mediate H3K36 methylation and CBP mediate H3K27 acetylation. H3K27 acetylation reinforces retention of the BAP/PBAP chromatin remodeling complex on promoters while the H3K36 methylation on the gene body is reinforced by Kismet. Thus, both histone modifiers and chromatin remodelers of trxG work synergistically to overcome the PcG mediated gene repression and mediate transcriptional activation. For simplicity, subunits and variants of complexes are not shown.

2.4.3 PcG and trxG antagonism

Both PcG and trxG antagonize each other which results in alteration of chromatin association and consequent chromatin modifications of one or the other group (Turner, 2002). For example, the PRC2 complex is directly inhibited by the trxG associated H3K4 and H3K36 methylation marks, as the catalytic activity of the PRC2

is significantly decreased on the nucleosomes carrying the two modifications (Schmitges et al., 2011). The SUZ12 subunit of PRC2 recognizes both marks to allosterically inhibit the catalytic subunit of PRC2 (Schmitges et al., 2011). Contrastingly, the core member of the dRAF complex, KDM2 is known to erase the H3K36 methylation (Lagarou et al., 2008). Additionally, KDM2 knockdown in *Drosophila* larvae leads to an increase in H3K4 methylation and was also suggested to be H3K4 demethylase (Kavi and Birchler, 2009). Another demethylase No66 is also targeted to H3K36 methylation by the PcG protein PCL3 (Brien et al., 2012). The PRC1 mediated ubiquitination of H2A is also known to inhibit the catalytic domain of the H3K36 methylation specific methyltransferases (Yuan et al., 2013). In addition, the chromatin remodeling complexes including SWI/SNF complexes are inhibited by the PRC1 (Francis et al., 2001). Thus it is PRC1 that plays the central role of PcG mediated gene silencing as suggested in recent reports (Blackledge et al., 2020; Cohen et al., 2020; Healy and Bracken, 2020; Tamburri et al., 2020; Zhao et al., 2020).

Acetylation and methylation of H3K27 residue are two other histone modifications that antagonize each other and are associated with trxG and PcG, respectively. CBP in TAC1 complex is known to acetylate H3K27 which in turn prevents H3K27 methylation by PRC2 (Tie et al., 2009, 2014). Conversely, the loss of SUZ12 has been shown to result in H3K27ac accumulation that suggests H3K27 methylation prevents acetyltransferases from catalyzing H3K27 acetylation (Pasini et al., 2010). As for erasing the H3K27me mark, a protein in COMPASS complex, Utx acts as demethylase specifically for H3K27 methylation (Agger et al., 2007; Lan et al., 2007; Min et al., 2007). On the other hand, H3K27ac mark is erased by NURD complex that facilitates PRC2 recruitment and H3K27me3 accumulation (Reynolds et al., 2012).

2.5 Cell signaling to chromatin

PcG and trxG occupy their target genes irrespective of their expression state (Beisel et al., 2007; Papp and Müller, 2006; Ringrose et al., 2004) and co-occupy the same loci on chromatin (Orlando et al., 1998; Strutt and Paro, 1997). Much work has been done to understand the molecular and biochemical nature of PcG mediated repression and mechanisms by which trxG antagonize it, yet the complex question of

how the decision of gene activation or repression is made remained largely unanswered. The cell signaling pathway components, especially kinases, may play a crucial role in this regard due to two reasons. Firstly, cell signaling pathways do respond to intra- and extracellular changes and secondly, due to their capacity to repress or activate target genes. This section will cover the general classification of kinases and the known junctures of cell signaling and PcG/trxG mediated cell memory.

2.5.1 Kinases and their classification

In most eukaryotes, only 1-4% of the genome encodes for kinases yet they alter more than two-third of the total proteins and their subsequent cellular functions by phosphorylation (Manning et al., 2002b, 2002a). In accordance with the significance of kinases, phosphorylation is the most common post-translational modification employed by nature to cope with the dynamic environment in cells and tissues in an organism (Ardito et al., 2017). Phosphorylation is a biochemical reaction, catalyzed by kinases, which involves the transfer of *γ*-phosphate from a nucleotide triphosphate molecule (mostly ATP) to a substrate molecule (Miller and Turk, 2018). Based on the amino acid residue to which the phosphate group is added, protein kinases belong to three general categories which are, the serine-threonine kinases, tyrosine kinases and dual-specificity kinases. The ratio between phosphoserine, phosphothreonine and phosphotyrosine inside cells is reported to be 48:7:1. In other words, phosphoserine is roughly 85% of total protein phosphorylations, the phosphothreonine constitute 12.5% and phosphotyrosine contributes only about 1.5% in all phosphorylations of proteins (Olsen et al., 2006).

Kinases control a wide range of biological processes ranging from cell cycle to regulation of metabolism by changing structures and functions of their substrates via phosphorylation (Roskoski, 2015). Kinases constitute a large group of proteins that are diverse in structure as well as in their biological functions. They are classified into different groups, families and subfamilies according to their sequence, structural similarity and evolutionary history (Manning et al., 2002a). These groups usually have common substrate site-specificity while the families are categorized based on the sequence and functional similarity. The classification system is continuously

evolving as originally kinases were divided into 5 groups (Hanks and Hunter, 1995) that are now extended to 11 groups. Gerard Manning, who initially worked with *Drosophila* as a genetic model organism for tubular organs development (Samakovlis et al., 1996), laid the foundation of modern classification scheme (Manning et al., 2002b, 2002a). General descriptions of different groups of kinases are given in **Table 2.7**.

Table 2.7: General description of each of the kinase groups.

Group	Rational behind Name	General description
AGC	Named after protein kinase A, G, C	Serine threonine kinases that have similar catalytic domain to cAMP-dependent protein kinase 1, cGMP-dependent protein kinase and protein kinase C
CMGC	Named after CDK, MAPK, GSK3 and CLK	CDKs are involved in cell cycle regulation. MAPK are cell signaling kinases that regulate cell proliferation, cell death, differentiation and other biological processes. GSK is involved in Wnt signaling pathway and glycogen metabolism, while CLK are involved in pre-mRNA processing.
CAMK	Named after Calmodulin regulated kinases	Kinases that are structurally similar to CAM kinases are grouped here. CAM kinases are serine-threonine kinases regulated by Calcium ion/calmodulin.
CK1	Named after casein kinase 1 but now called cell kinases 1	Structurally very similar within the group, but very distinctive from other kinases that phosphorylate serine-threonine residues. This group includes regulators of different signaling pathways, DNA repair and transcription.
STE	Named after STE 7,11,20	Three families of kinases that sequentially activate each other in MAP kinase signaling cascade. STE20 proteins phosphorylate STE11 that in turn, phosphorylate STE7. STE7 eventually phosphorylates MAP kinases.
TK	tyrosine kinases	Kinases that only phosphorylate tyrosine residues and are relatively new in evolutionary history. TK group members are only present in higher order eukaryotes and function as cell surface receptors or in close vicinity to cell membranes.
TKL	tyrosine kinases like	A diverse group of kinases that are related to tyrosine kinases but not specific for phosphorylation of tyrosine resides. Around half of plant kinome code for members of this group.
RGC	Receptor Guanylate Cyclases	Receptor guanylate cyclases are structurally unique single pass transmembrane receptor proteins. They have a dead kinase domain on the intracellular side.
PKL	PKL fold containing	Another diverse group of kinases, that share a common PKL fold in structure and mechanism of catalysis. Apart from protein kinases, this group also covers several lipids, sugar and other small molecule kinases.

Continued from previous page		
Atypical	known and predicted kinases with no kinase domain	Kinases that lack kinase domain but experimentally their kinase activity is shown. Some of this group members are only predicted to be kinase because of their homology with other atypical kinases. The kinase activity of many of the kinases of this group is supported by small number of reports, so it can be possible that such kinases are false positive.
Others	Kinases that do not fit in conventional kinase groups	Structurally so diverse that they cannot be grouped in any of the other groups but covers many important kinases. This group contains families of Aurora, Bub, Haspin, Wee kinases and others.

2.5.2 Known junctures of cell signaling and cell memory

Cell signaling pathways exert tight control over gene regulatory mechanisms of chromatin by either directly phosphorylating histones or by phosphorylating PcG and trxG proteins. Both chromatin-associated kinases and the kinases that modify PcG and trxG proteins contribute to the ability of cells to sense and respond to alteration in the cellular microenvironment.

2.5.2.1 Phosphorylation of PcG and trxG proteins

Phosphorylation of many PcG and trxG proteins are known to alter their interaction with histones, subcellular localization and enzymatic activity. Such alterations can positively as well as negatively affect the functioning of the proteins. For example, the EZH2 is known to be phosphorylated by AKT (Cha et al., 2005), JAK3 (Yan et al., 2016), AMPK (Wan et al., 2018), GSK3β (Ko et al., 2016), p38 (Anwar et al., 2018), CDK1 and CDK2 (Chen et al., 2010; Wei et al., 2011; Yang et al., 2015; Zeng et al., 2011) at unique sites to alter EZH2 functionality differently. EZH2 is translocated to the cytoplasm as a result of p38 mediated phosphorylation at threonine 367 position (Anwar et al., 2018), while its phosphorylation by AKT at serine 21 reduces its interaction with histone H3 (Cha et al., 2005). On the other hand, two cyclin-dependent kinases CDK1 and CDK2 are known to phosphorylate EZH2 to either decrease its methyltransferase activity or to maintain H3K27me3 mark in daughter cells during mitosis (Chen et al., 2010; Kaneko et al., 2010; Zeng et al., 2011). Similarly, the phosphorylation of another core member of PRC2, Esc, is also known to inhibit the formation of PRC2 (Ng et al., 2000; Tie et al., 2005).

In the case of PRC1, Bmi1 (PCGF4), a mammalian homolog of Psc is known to be phosphorylated by AKT leading to enhanced enzymatic activity of PRC1 (Nacerddine et al., 2012). In contrast, Bmi1 is also phosphorylated by MK3 of MAPK signaling pathway to reduce its binding ability to chromatin (Voncken et al., 2005). Another Psc mammalian homolog, Mel18 (PCGF2) is also phosphorylated and, as a result, the ubiquitination activity of the Mel18/Ring1B complex is reported to be increased (Elderkin et al., 2007). Moreover, one of the Polycomb homologs in humans, CBX7 is known to be phosphorylated through MAPK signaling pathway. This particular phosphorylation enhanced the gene repression mediated by CBX7 (Wu et al., 2013). The phosphorylation of CBX2 is also known to enhance its binding specificity for H3K27me3 (Hatano et al., 2010). CK2 is found to be responsible for CBX2 phosphorylation and the mutant CBX2, that lacks phosphorylation site, failed to repress p21, a target gene of PRC1 (Kawaguchi et al., 2017). Kinases not only modify PcG proteins but also regulate them at the transcriptional level. For example, the c-Jun kinase of the JNK signaling pathway is known to suppress PcG proteins at the transcriptional level in flies (Lee et al., 2005; Roumengous et al., 2017). Just like PcG, function of trxG proteins is also effected upon their phosphorylation. One such example is the phosphorylation of BAP60c of the SWI/SNF complex by p38 that results in the formation of a larger complex containing MyoD and SWI/SNF components. The formation of such a complex contributes to the activation of MyoD target genes (Forcales et al., 2012; Simone et al., 2004). The COMPASS complexes recruitment to the target genes during differentiation are shown to be under the control of p38 MAPK pathway by controlling the recruitment of Ash2L, a core part of COMPASS complexes (Rampalli et al., 2007).

2.5.2.2 Phosphorylation of histone residues

Histone modification is one of the earliest discovered mechanisms involved in cell memory when histone acetylation was suggested to be linked with gene activity (Allfrey et al., 1964). Phosphorylation of histones was discovered in the mid-1960s (Ord and Stocken, 1966) and is known to associate with histone acetylation in cases of phosphorylated histone H3S10 (Lo et al., 2000), H3T11 (Clements et al., 2003) and H3S28 (Zhong et al., 2003). The most extensively studied histone phosphorylation

mark is probably the H3S10p that is known to be placed by different kinases including Aurora B (Hsu et al., 2000), IKK (Yumi et al., 2003), Rsk2 (Sassone-Corsi et al., 1999), MSK1 (Wang et al., 2001) and AKT (He et al., 2003). Several enzymes have been shown to cause the phosphorylation of the same histone residues that make deciphering the context-dependent roles of phosphorylation marks challenging. Cells stimulated by EGF signaling have shown H3S10p association with H3K9ac and H3K14ac, marks linked to gene activation (Cheung et al., 2000; Clayton et al., 2000). The association of H3S10p with histone acetylation can be due to its recognition by Gcn5 acetyltransferase of the SAGA complex (Lo et al., 2000). The histone code of H3K9ac/H3S10p/H3K14ac has been shown to provide a platform for BRD4, homolog of Fs(1)h, binding to chromatin (Zippo et al., 2009). Similar to H3S10p, H3T11p mark also enhances the interaction of Gcn5 with promoters of its target genes (Clements et al., 2003). H3T11p mark also assists in the removal of repressive H3K9 methylation mark by a demethylase KDM4C (Metzger et al., 2008). Contrastingly, phosphorylation of histone H3T6 is known to inhibit demethylase LSD1 from acting on methylated H3K4, thus prevent removal of active methyl mark placed by COMPASS complexes. The kinase responsible for H3T6p is PKCβ1 (Metzger et al., 2010) and for H3T11 is PRK1 (Metzger et al., 2008) and both phosphorylations occur in response to androgen receptor induced signaling (Metzger et al., 2008, 2010).

Not only is serine/threonine phosphorylation crucial in providing plasticity to cell memory mechanisms, but phosphorylation of tyrosine residues is also known to effect transcription. For example, phosphorylation of H2B at tyrosine 37 (H2BY37p) by Wee1 is known to suppress transcription of core histone genes by excluding RNA Pol-II and a transcription factor, NPAT (Mahajan et al., 2012). Another example is the phosphorylation of H3Y41 by JAK2, known to disrupt the chromatin binding of the HP1α protein. The HP1α interacts with H3 core via its chromo shadow domain that is disrupted by addition of phosphate group to H3Y41 (Dawson et al., 2009). HP1 is also dissociated from chromatin during mitosis as a consequence of phosphorylation of adjacent H3S10 (Hirota et al., 2005), thus the H3S10p/H3K9me has long been proposed as a binary switch (Fischle et al., 2003) between gene activation and silencing. A similar switch can be present at H3T3p/H3K4me and H3S28p/H3K27me,

as H3T3 phosphorylation is known to inhibit binding of TFIID (Varier et al., 2010), CHD1 (Flanagan et al., 2005) and MLL5 (Ali et al., 2013) to H3K4me3. H3T3p also protects H3K4me3 from KDM4A demethylase *in vitro* (Su et al., 2016). Similarly, H3S28p is known to displace PcG proteins from H3K27me3 mark (Gehani et al., 2010; Lau and Cheung, 2011) but at the same time, it protects the H3K27me3 mark from demethylases (Kruidenier et al., 2012; Sengoku and Yokoyama, 2011). However, little is known about two additional phospho-methyl switches namely H3T22p/H3K23me and H3K79me/H3T80p. Phosphorylation on H3T22 has also been shown to negatively affect KDM4A demethylase binding to H3K23 methylation (Su et al., 2016) but H3T22 phosphorylation is yet to be established as a modification present *in vivo*. On the other hand, H3T80p is an established mark (Vermeulen et al., 2010) but it is not known whether it negatively regulates the recognition of H3K79 methylation by proteins. Nonetheless, structure to function analysis of yeast homolog of Dot1, a methyltransferase responsible for H3K79 methylation, has suggested that a negatively charged phosphate group on H3T80 may affect its enzymatic activity (Sawada et al., 2004). Additionally, H3S28p, together with H3S10p, is known to initiate remodeling of the promoter region via SWI/SNF remodelers in response to MAPK signaling (Drobic et al., 2010). The chromatin binding of Ph and Pc in *Drosophila* is also controlled by H3S28p during mitosis (Fonseca et al., 2012). Interestingly, the PRC1 mediated H2A ubiquitination also neighbors a threonine that is also known to be phosphorylated by Ballchen (Aihara et al., 2004), a protein that has been characterized as a novel regulator of trxG mediated gene activation in this dissertation. **Figure 2.2** summarizes the effects of histone phosphorylation on cell memory associated histone modifications.

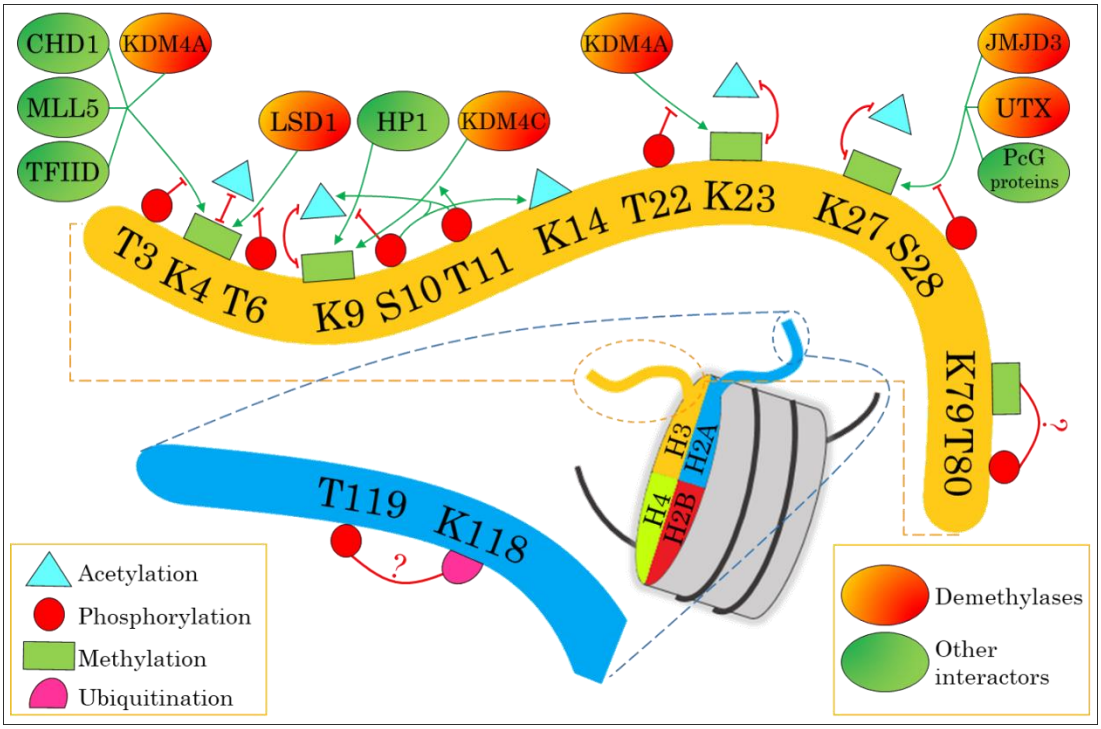

Figure 2.2: Role of histone phosphorylation in cell memory. Histone phosphorylation can positively and negatively regulate the binding of proteins involved in cell memory. The H3T3p mark acts as a switch for preventing proteins from recognizing H3K4 methylation. H3S10p inhibits binding of HP1 to H3K9 methylation mark and H3S28p also prevents PcG proteins from recognizing H3K27 methylation. Histone phosphorylations inhibit or promote other histone marks by both preventing and stimulating erasers. H3T3p, H3T6p, H3T22p and H3S28p protect neighboring methylation marks from demethylases (red-orange ovals) but in contrast, H3T11p promote demethylation of H3K9 by KDM4C. Histone phosphorylations also effect writer proteins to change histone marks landscape. H3T11p and H3S10p are recognized by Gcn5 acetyltransferase and associated with histone acetylation marks. MLL5 and writers from PcG proteins are also prevented from recognizing H3K4 and H3K27 methylation marks by H3T3p and H3S28p, respectively. Histone phosphorylations severely impact neighboring histone marks but the effects of H3T80p on neighboring H3K79 methylation and H2AT119p on neighboring ubiquitination mark are yet to be deciphered.

2.6 Quest for a link between cell signaling and trxG

How signaling to chromatin works in maintenance of cell type specific gene expression patterns remains an enigma. Despite discovery of well characterized developmental cell signaling pathways as well as epigenetics factors and mechanisms, the gulf between epigenetics and cell signaling remains wide. Interestingly, while forward genetic screens were very successful in discovering a large number of PcG and trxG genes, they failed to develop a link between cell memory and cell signaling. The reverse genetics approach using genome-wide RNA interference (RNAi) screens provides an opportunity to analyze the phenotypic

consequences of all possible candidates in a high-throughput assay. Since RNAi leads to reduced gene dosage due to knockdown effect as compared to complete loss of function mutations, the subtle phenotypic consequence of signaling genes on chromatin may be elucidated in the presence of a highly sensitive reporter assay. During this dissertation, I aimed to elucidate a link between trxG mediated gene activation and cell signaling by employing a high throughput RNAi approach against known kinases and cell signaling genes in *Drosophila*. This section covers the review of the reported studies that deciphered different biological processes and cellular pathways employing RNAi screens. The focus will be on studies done using *Drosophila* as a model system.

2.6.1 *In vivo* RNAi screens

In *Drosophila*, various approaches have been developed to perform RNAi screens both *in vivo* and *ex vivo. In vivo* RNAi screens in *Drosophila* has been the source for elucidating several biological processes, such as female reproductive behavior (Yapici et al., 2008), immunity to bacterial infection (Cronin et al., 2009), development of sensory organs (Mummery-Widmer et al., 2009), neuronal-specific glycosylation (Yamamoto-Hino et al., 2010), genes involved in heart function (Neely et al., 2010), nociception (Garrity et al., 2010), obesity (Pospisilik et al., 2010), maintenance of neural stem cells (Neumüller et al., 2011), neuromuscular synapse formation (Valakh et al., 2012), development of wing (Reim et al., 2014), and the female germline cells development (Yan et al., 2014). However, such screens need a readout that can be challenging in a living organism. Observing a specific phenotype *in vivo*, that can serve as the readout for a biological process of interest, mostly demands complex crossing schemes, laborious dissections or costly assays, hindering the efficiency of such screens.

2.6.2 *Ex vivo* RNAi screens

Performing an RNAi screen is more convenient in *Drosophila* cell lines as compared to mammalian cells due to their ability to absorb dsRNA from culture media. To knock down a specific gene effectively, *Drosophila* cells, which use scavenger receptors for taking up dsRNA (Ulvila et al., 2006), are simply bathed with dsRNA against a gene (Echeverri and Perrimon, 2006). During my Ph.D., I particularly took

advantage of this trait of *Drosophila* cell culture to perform a kinome-wide RNAi screen to discover cell signaling genes linked to maintenance of gene activation by trxG. Other advantages of using *Drosophila* as a model system relevant to this dissertation are described in the **Appendix A**. Although 500-1000 base pair long dsRNA fragment increases the efficiency of knockdown compared to single siRNA (Haley et al., 2010; Somma et al., 2002), it can also lead to off-target knockdown of unintended genes. Readouts for monitoring effect of knockdown in *ex vivo* RNAi screens are more convenient for researchers when compared with readouts of *in vivo* screens. Imaging of morphology of cells, biochemical assays, imaging of tagged proteins and FACS assays (Heigwer et al., 2018) are some of the examples of such readouts. In this particular study, a well-characterized *Ultrabithorax PRE* regulated reporter gene assay was used as a readout for kinome-wide RNAi screen (Umer et al., 2019).

2.6.2.1 Reporter gene assays

A reporter gene that is responsive to the biological process of interest is transfected in cells treated with dsRNA in this type of readout (Nybakken et al., 2005). To make a typical reporter gene construct, a fluorescent protein or luciferase coding sequence is cloned under the pathway-specific promoter of the biological process of interest. Another common strategy for this purpose is fusing the coding sequence of a reporter gene with a pathway-specific protein. *Ex vivo* RNAi screens in *Drosophila* cells, using reporter genes as readout, have helped to understand various biological pathways including JAK/STAT signaling pathway (Baeg et al., 2005; Müller et al., 2005), Wg/Wnt signaling pathway (Bartscherer et al., 2006; DasGupta et al., 2007), Hedgehog signaling pathway (Lum et al., 2003; Nybakken et al., 2005), NF-kB signaling pathway (Foley and O'Farrell, 2004; Gesellchen et al., 2005) and gut immunity (Goto and Kiyono, 2011). Interestingly, screens for deciphering RNAi mechanism of action were also performed using reporter gene readout using *Drosophila* cell culture (Dorner et al., 2006; Eulalio et al., 2007). The reporter gene is usually introduced into cells during a high-throughput screen by either using a stable cell line expressing reporter or by adding a transfection step into the screen. In high-throughput screens, cells treated with dsRNA against genes that affect viability of

the cells or effecting general transcriptional machinery can give false positive results. To cope with the false positives, usually a second reporter is also delivered into the cells which is unrelated to the pathway of interest. For example, a construct constitutively expressing *Renilla* luciferase can be used with *Firefly* luciferase reporter to normalize the effects of knockdown which are not relevant to the biological process of interest (Heigwer et al., 2018). I used a previously characterized *PBX-bxd-IDE-F.Luc (PRE-F.Luc)* reporter in which *Firefly* luciferase reporter is expressed under the control of *Ubx* promoter and minimal *bxd* PRE (Fritsch et al., 1999; Umer et al., 2019). Besides, *PBX* (*postbithorax*) (Zhang et al., 1991) and *IDE* (*Imaginal Disc Enhancer*) (Christen and Bienz, 1994) enhancers are also in the reporter to enhance its robustness, thus named *PBX-bxd-IDE-F.Luc (PRE-F.Luc)*. The same construct but with *LacZ* reporter gene has been used *in vivo* to show effect on DNA binding of a PcG protein, Pleiohomeotic, upon point mutations in the PRE (Fritsch et al., 1999). For efficient and robust use in a high throughput *ex vivo* screen *LacZ* reporter gene was replaced with *Firefly luciferase* gene in our lab (Umer et al., 2019). A construct, constitutively expressing *Renilla luciferase* was also employed along with the reporter to normalize the non-specific effects of knockdown.

2.6.2.2 RNAi libraries for *ex vivo* screens

For high throughput screens in *Drosophila* cells, dsRNA libraries were required to knockdown large sets of genes in parallel. Various dsRNA libraries have been developed for this purpose in the past few years including DRSC, HD2 and HD3. DRSC is a publically available source for dsRNA reagents provided by Harvard *Drosophila* RNAi Screening Center. DRSC 2.0 has dsRNAs against 13,900 genes arrayed on 66 plates (384-well) (Ramadan et al., 2007). This library uses one or two dsRNA against each gene that are optimized for high efficiency of knockdown or for no off-target effect, making it a library of choice for most researchers. Apart from DRSC, Heidelberg 2 (HD2) and Heidelberg 3 (HD3) are two other dsRNA libraries developed in Michael Boutros lab (Billmann et al., 2016; Horn et al., 2010) that contain one or two independent dsRNA against 14,334 unique genes. HD3 is the latest of the dsRNA libraries that covers two dsRNA molecules, designed with optimization for high degree of specificity. Both DRSC and HD libraries are genome-wide libraries, but

several smaller libraries (covering kinases/ phosphatases/ transcription factors) have also been derived from these libraries. HD2 kinome-wide sub-library is the one that has been utilized during this study that covers >400 genes coding for all known and predicted kinases, phosphatases and associated proteins (Heigwer et al., 2018). During this dissertation, HD2 kinome-wide RNAi library was used which led to discovery of known kinases and cell signaling genes which affect *PRE-Luc* reporter similar to the effect of depletion of trxG genes. Moreover, I specifically characterized Ballchen (Ball) which is a serine-threonine protein kinase and was among the top candidates in my screen.

2.7 Known functions of Ball

Ball belongs to a small but highly conserved family of VRK (vaccinia related kinase) from the CK1 group of kinases. The conserved portion of Ball with respect to other VRK kinases, is restricted only to the kinase domain (Aihara et al., 2004). In *Drosophila*, another gene CG8878 has 36% sequence identity to Ball but 100% pupal lethality of *ball* null mutant flies suggest that Ball has important non-redundant functions in *Drosophila* (1998a). *ball* gene has two annotated transcripts but how they function exclusively is not yet known. Both transcripts translate into the same 65.9kDa protein that is 599 amino acid long. This gene comprises of 2203bp of genomic sequence at cytological position 97D on the right arm of the third chromosome (1998a).

Ball has been shown to have a high affinity for chromatin and it phosphorylates threonine 119 at the C-terminus of histone H2A (Aihara et al., 2004), thus also known as nucleosomal histone kinase 1 (NHK-1). H2A phosphorylation that is a target of Ball activity is required for acetylation of histone H3 and H4 in meiosis (Ivanovska et al., 2005). Immunostainings of Ball has shown that it is on chromatin during mitosis but in the cytoplasm during S phase (Aihara et al., 2004). Mutation in *ball* results in female sterility due to defects in the formation of meiotic chromosomal structures. It has been shown that *ball* mutation also affects mitosis (Ivanovska et al., 2005) as karyosome, an important prophase I structure, needs Ball kinase activity (Fiona Cullen et al., 2005; Ivanovska et al., 2005). Phosphorylation of BAF (Barrier to Autointegration Factor) by Ball is of key importance during

karyosome formation as the BAF function is to link the chromatin to the nuclear envelope. If BAF is not phosphorylated, the ectopic association of chromosomes with the nuclear envelope in oocytes occurs in absence of Ball. The aforementioned studies suggest that Ball regulates chromosome anchorage to nuclear envelope thus encourages the formation of karyosome (Lancaster et al., 2007).

Phosphorylation of histone H2A at threonine 119 position (H2AT119p) that is the target of Ball kinase activity is an important mark for cell cycle progression as its spatio-temporal patterns are controlled by multiple mitotic kinases (Brittle et al., 2007). H2AT119p is enriched at centromeres during mitosis and Aurora B kinase complex is essential for this enrichment. If Polo kinase is depleted, this mark is up-regulated on chromosome arms in mitosis, suggesting that Polo kinase negatively regulates functions upstream of Ball (Brittle et al., 2007). Another role of Ball is to maintain the self-renewing capability of male and female germline stem cells (Herzig et al., 2014). It was shown that Ball is also required to maintain the self-renewing ability of neuronal stem cells. *ball* mutant larvae have a minute brain which is due to reduced proliferation rate of neuroblasts (Yakulov et al., 2014).

In *Drosophila* S2 cells, *ball* knockdown led to the conclusion that it is required for mitotic progression (Fiona Cullen et al., 2005). Ball itself is also phosphorylated during mitosis and female meiosis which hints towards its link to some kind of regulatory system of mitosis and meiosis (Fiona Cullen et al., 2005) but the upstream factors that lead to Ball phosphorylation are not yet known. Nonetheless, DNA breaks induced by x-rays, are known to suppress Ball kinase activity in cultured cells. Similarly, the presence of unrepaired DNA breaks in oocytes have also shown to reduce the Ball dependent nuclear events (Lancaster et al., 2010). These results suggest that Ball is acting as a link between DNA repair machinery and cell cycle machinery of the cell and halting cell cycle if the DNA needs to be repaired (Lancaster et al., 2010).

As already mentioned, Ball belongs to the VRK (Vaccinia related kinases) family of kinases. Although bacteria and yeast don't have any VRK protein, *C. elegans* has one kinase, named VRK1, that belongs to the VRK family (1998b). VRK proteins are named so due to their homology with B1R kinase of vaccinia viruses. The mammalian

genome encodes three members of VRK family that are named VRK1, VRK2 and VRK3 but only VRK1 has been extensively studied (Campillo-Marcos and Lazo, 2018). VRK2 has a transmembrane domain in it and usually resides on endoplasmic reticulum and mitochondrial membranes (Blanco et al., 2006). Although VRK3 has a nuclear localization signal, it has no catalytic activity (Nichols and Traktman, 2004) and is believed to play a role in the regulation of phosphatases or might function as a scaffold protein (Kang and Kim, 2006). VRK2 and VRK3 are proteins that are less characterized and are among a list of understudied kinases generated by IDG (Illuminating the Druggable Genome) program of NIH (National Institutes of Health).

The well-studied mammalian VRK1 is a nuclear kinase that resides on chromatin but gets excluded from chromosomes during their condensation in late mitosis. VRK1 is known to interact with different transcription factors, histone proteins and other proteins of the cell cycle and DNA repair machinery. The histone substrates for VRK1 include H3 (Kang et al., 2007), H2A (Aihara et al., 2016), H2AX (Salzano et al., 2015) and macroH2A1 (Kim et al., 2012) while the transcription factors that are phosphorylated by VRK1 are p53 (Vega et al., 2004), Sox2 (Moura et al., 2016), c-Jun (Sevilla et al., 2004a), ATF2 (Sevilla et al., 2004b), CREB (Kang et al., 2008) and FXR (farnesoid X receptor) (Hashiguchi et al., 2016). Like *Drosophila* Ball, VRK1 also phosphorylates BAF1, thus affecting nuclear membrane structure (Nichols et al., 2006).

The upstream signaling to VRK1 for regulating its activity includes its interaction with Ran GTPase (Sanz-García et al., 2008). Ran protein bound to GTP (Guanine triphosphate) physically interacts with VRK1 but it does not inhibit VRK1 activity in this state. Once the bound GTP is converted into GDP (Guanine diphosphate), through the GTPase activity of Ran, VRK1 is inhibited. The GEF (Guanine exchange factor) of Ran forms the Ran-GTP gradient with a high concentration near chromosomes during mitosis, thus VRK1 is as restricted as it is away from chromosomes (Kaláb et al., 2006). VRK1 has also been shown to function downstream of Plk3 (Polo-like kinase 3) during mitosis. Plk3 upregulates VRK1 by phosphorylating it at serine 340 position (Lopez-Sanchez et al., 2009). VRK1 is also auto-regulated by some of its substrate proteins. Sox2 and p53 are two such

examples, where the *vrk1* gene is downregulated by phosphorylated p53 and upregulated by Sox2. By phosphorylating p53 at threonine 18, VRK1 stabilizes it, which in turn downregulates VRK1 gene expression (Valbuena et al., 2006). On the other hand, Sox2 protein work synergistically with VRK1 but its gene is downregulated by the accumulation of VRK1 protein (Moura et al., 2016), thus both Sox2 and p53 interactions with VRK1 forms auto-regulatory loops. Histone macroH2A1 also has been shown to inhibit VRK1 kinase activity by direct physical interaction (Kim et al., 2012). Despite wide-ranging information known about Ball, genetic and molecular link between PcG/trxG system and Ball is not yet known.

3 METHODOLOGY

3.1 Kinome-wide RNAi screen

PCR amplification of the templates for the preparation of dsRNAs was done using T7-tailed oligonucleotides as primers. *In vitro* transcription of the templates was done with T7 Megascript kit according to the manufacturer's instructions (Ambion). Primers for all the known members of trxG were chosen from the HD2 library (Heidelberg 2) (Horn et al., 2010). Complete primer and amplicon sequence information can be found at http://rnai.dkfz.de.

Once the dsRNAs were prepared, bathing of D.Mel-2 cells with dsRNAs against the whole kinome was carried out. Kinome-wide RNAi screen was performed in duplicates and each replicate contains three wells of 384 well plate with dsRNAs against a specific gene. The controls described in **Results Section 4.1** were present in each plate in triplicates. Six 384 well plates were used for each of the two duplicates. The final concentration of RNA used per well was 50ng/µl. Cells were grown to approximately 80% confluency and counted with a hemocytometer. 8000 cells in 30µl were dispensed per well with a multidrop dispenser. The cells were spun down for 10s at 900 rpm and the plates were sealed and incubated at 25^{o}C. Next day, transfection with *PRE-F.Luc* and *Actin-R.Luc* was done, details of which are given in **Appendix B**. Five days later, F.Luc and R.Luc values were recorded using a dual luminescence reader (Boutros et al., 2004). The ratios of the experimental reporter F.Luc to the invariant co-reporter R.Luc values was calculated to exclude possible artifacts and exclude genes that may affect general transcription. Knockdown of genes that affected both F.Luc and R.Luc were removed from further analysis. Relative F.Luc expressions were averaged for both replicates. The primary kinome-wide RNAi was carried out by Dr. Saima Anwar in Michael Boutros lab (DKFZ, Heidelberg) and the results were analyzed in collaboration with Mithani Lab (Department of Biology, LUMS). Instead of using 99.99% confidence values as cut-offs, more stringent cut-offs were defined based on *trx* and *ash1* Z-scores (higher Z-score). The whole kinome-wide RNAi screen procedure is summarized in **Figure 3.1** and the list of all analyzed genes is given in **Appendix H.**

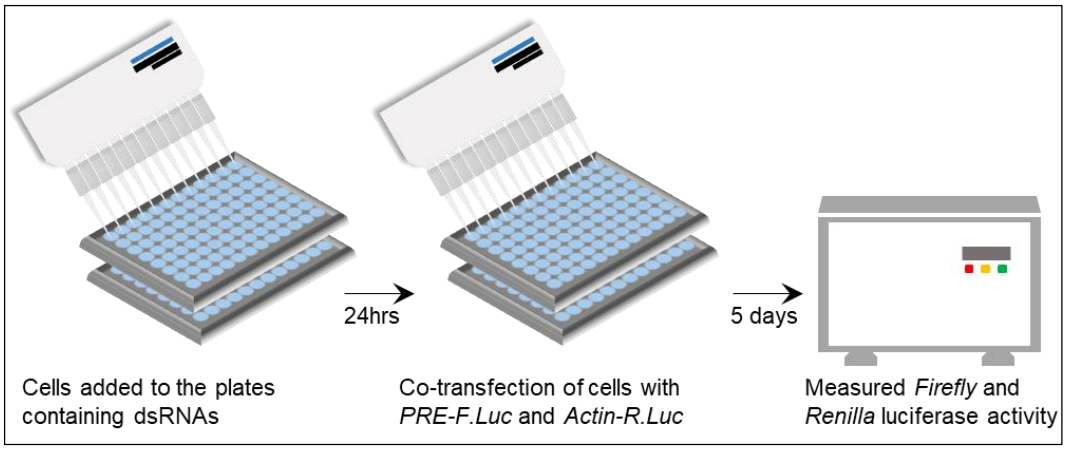

Figure 3.1: Schematics of kinome-wide RNAi screen. 384 well plates containing dsRNAs for different genes were loaded with equal number of cells in each well. 24 hours after seeding cells, *PRE-F.Luc* and *Actin-R.Luc* were co-transfected and luciferase values were determined five days later.

3.2 Secondary RNAi screen

The dsRNAs were prepared as described in the previous section but the primers for amplification of the templates were chosen from DRSC library instead of HD2 library. Complete primer and amplicon sequence information can be found at https://fgr.hms.harvard.edu/fly-cell-based-rnai. Once the dsRNAs were prepared the same procedure as mentioned in previous section was repeated with minor changes that are following. Instead of 384 well plates, 96 well plates were used with each well having 2µg RNA. 50000 cells in 100µl were dispensed in each well. The transfection mixture preparation is given in **Appendix B**.

3.3 Fly genetics

3.3.1 List of fly strains

The fly strains used during this study are listed in four tables. The strains used for genetic analysis of *ball* mutants, the strains used for the generating somatic clones, the strains generated through genetic crosses during this study for somatic clones and the GAL4 line and RNAi lines are given in **Table 7.1**, **Table 7.2**, **Table 7.3** and **Table 7.4**, respectively in **Appendix G**.

3.3.2 Genetic analysis of *ball*^{2} mutant

Mutant fly line of *ballchen* (*ball*^{2}) was crossed with two alleles of *Polycomb* (*Pc*^{1} and *Pc*^{XL5}) and also with two alleles of *trithorax* (*trx*^{1} and *trx*^{E2}). Details of all alleles are given in **Table 7.1**. *Polycomb* mutant flies crossed with *w*^{1118} and *trx* mutant flies

Table 3.1: Based on the severity of extra sex comb phenotype, flies were categorized and specific criterion was used to specify flies to different categories.

Categories	Scoring Criteria
-	no extra sex combs
+	1-2 hairs on second leg
++	more than 3 hairs on second leg
+++	more than 3 hairs on second leg and 1-2 hairs on third leg
++++	strong sex combs on both second and third pairs of legs

crossed with w^{1118} served as respective controls. All crosses were set up in triplicates and were given same conditions (25°C temperature and flipping to new vials after every 48 hours). For analysis of extra-sex comb phenotype, flies were categorized in 5 classes that are explained in **Table 3.1**. ~200 male flies with desired genotype from each cross were scored.

3.3.3 Crossing schemes

3.3.3.1 Crossing scheme for generation of somatic clones

To generate somatic clones for *ball*^{2} mutation, four fly lines were used in four subsequent crosses. All crosses were set at 25°C according to the crossing scheme described in **Figure 3.2**. Three stable fly lines are generated in this crossing scheme given in **Appendix G: Table 7.3** while using the fly lines listed in **Appendix G: Table 7.2**. For generating somatic clones, *HsFlp; +; P{neoFRT}82B, P{Ubi-GFP}* was crossed with *+; +; P{neoFRT}82B, ball*^{2}*/Tb* to obtain larvae of desired genotype. Once such larvae reach the age of 55 hours, they are given heat shock at 38°C for one hour. 65 hours after heatshock, the larvae with desired phenotype were dissected in 1X PBS (**Appendix B**).

3.3.3.2 Crossing scheme for homozygous *ball*^{2} embryos selection

To select homozygous *ball*^{2} embryos, *ball*^{2} mutation was balanced with a balancer expressing GFP under *Actin* promoter details of which are given in **Appendix G: Table 7.4**. This cross led to three classes of progeny which are; GFP expressing heterozygous embryos, balancer containing embryos and the homozygous *ball*^{2} mutant embryos with no GFP expression. Homozygous *ball*^{2} mutant embryos were the required ones for the experiments described in the **Results Section 4.6.3**. After egg-laying, 20-22 hours old embryos were used for quantitative PCR analysis, while

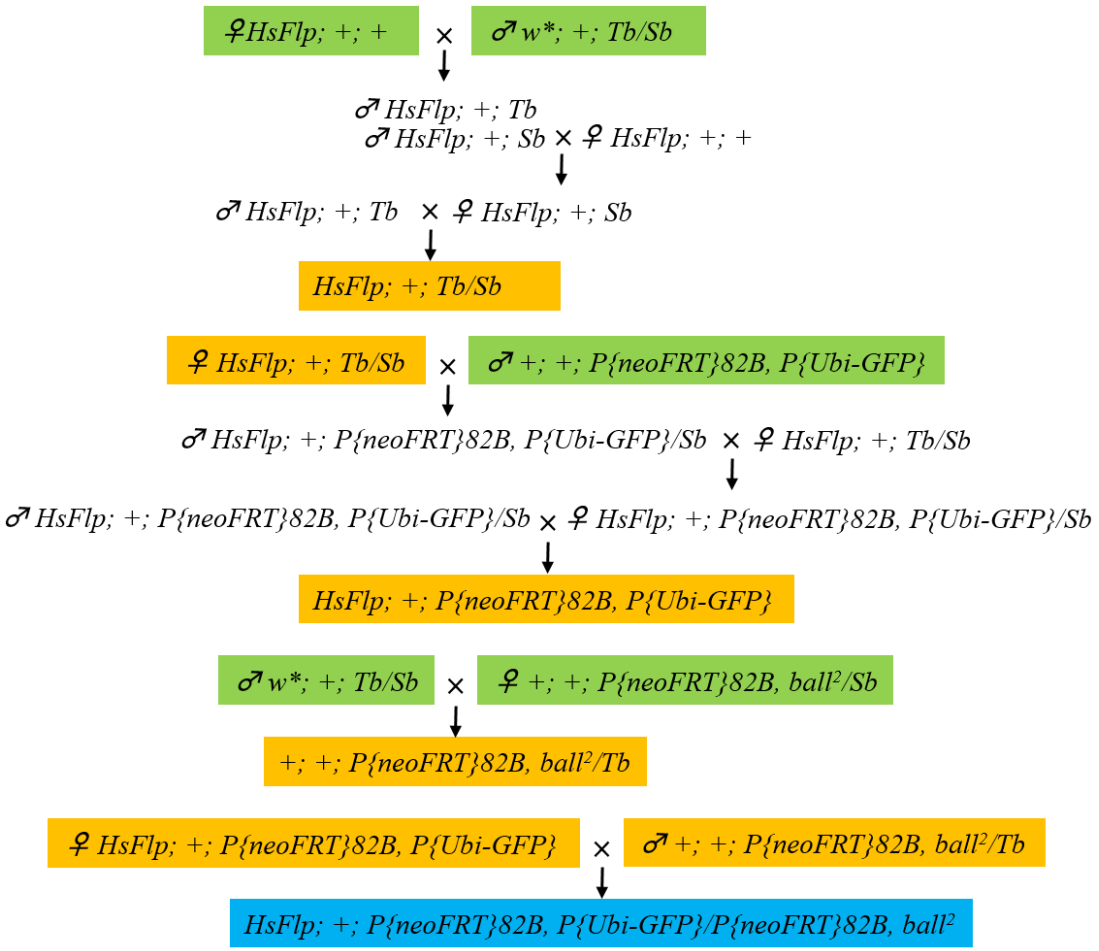

Figure 3.2: Scheme of genetic crosses to obtain *ball* flies of desired genotypes to generate somatic clones of *ball*^{2}. Complete crossing scheme that is used to generate larvae with *ball*^{2} homozygous somatic clones is depicted. Green are the fly lines used as primary source while orange are the fly lines generated in this procedure. Blue is the genotype of the larvae that were used to finally generate somatic clones.

12-14 hours old embryos were used for immunostainings. The experiment for the relative expression analysis was performed in triplicates while the immunostainings experiments were performed twice.

3.4 Immunostaining procedures

3.4.1 Immunostaining of embryos

For immunostaining of embryos, 12-14 hours old embryos were dechorionated in 3% sodium hypochlorite by incubating for 2-3 minutes. The homozygous *ball*^{2} embryos were selected under an epifluorescent stereomicroscope (Nikon, C-DSS230) on the bases of absence of GFP. 12-14 hours old *w*^{1118} embryos were used as control and the following procedure was used for both types of embryos simultaneously.

After selection of embryos with desired genotypes, embryos were transferred in a glass container that has 5ml heptane in it. To fix the embryos, 5ml of 3.7% formaldehyde solution made in PEM buffer (**Appendix B**) was added in the glass container and mixed by vigorous shaking. The solution was allowed to settle down for 20 minutes. Once the solution was settled, the formaldehyde (lower layer) was removed and 5ml methanol was added. The glass container was again vigorously shaken and the heptane (upper layer) was removed from it before filling the container with methanol. At this stage, embryos were given overnight incubation at 4^{o}C in methanol. Next morning, embryos were transferred in microcentrifuge tube and methanol was removed, followed by two washings with 1X PBS solution. After washings, embryos were blocked in 4% formaldehyde at room temperature on *WiseMix® RT-10* Digital Feedback Controlled Rotator with rotation of 25 rpm for an hour. Finally, embryos were transferred to a PCR tube and washed again with 1X PBS twice, followed by incubating embryos with specific primary antibody (see **Section 3.11**) overnight at 4^{o}C with 25 rpm on rotator. Next morning, embryos were again washed with 1X PBS twice and then incubated in DAPI and secondary antibody for three hours at room temperature on rotator set at 25 rpm. Finally, embryos were washed thrice with 1X PBS and after third wash, PBS was removed and Fluoromount-G® Mounting Medium was added to the embryos. With a wide pipette tip, embryos were transferred to glass slide along with the mounting medium and a coverslip was placed on them. The microscopy details are given in **Section 3.10.**

3.4.2 Immunostaining of imaginal discs

For immunostaining of imaginal discs, larvae were dissected using the inside-out method as it results in less loss as well as less damage to the imaginal discs. Larvae were first split into half with the help of fine forceps. The anterior half of the larvae was turned inside out by holding the anterior most end with one forcep which was passed through second forcep holding cut-end of larval carcass bringing inside of larval body out. Once the cuticle turned inside out, it was placed in a microcentrifuge tube containing 1X PBS. Around 30-40 larvae were dissected and placed in PBS within time window of half-hour. Next, PBS was removed and fixative solution (4% formaldehyde) was filled in the microcentrifuge tube. The microcentrifuge tube was

placed on rotator at 25 rpm for 30 minutes on room temperature. Once fixation was done, larvae were washed thrice with 1X PBS. After washings, 200µl blocking solution (**Appendix B**) was filled in the microcentrifuge tube. All of the inside out larvae were then transferred in a PCR tube. 200µl of primary antibody dilution made in Buffer 1 (**Appendix B**) was added in the tube and placed on rotator at 25 rpm at 4^{o}C overnight. Next morning, cuticles were washed twice with 1X PBS before adding 200µl mixture of DAPI and secondary antibody (see **Section 3.11**) and placed on rotation under aforementioned conditions for 2 hours. Finally, these cuticles were washed with PBS thrice (3X), each washing for 15 minutes, before dissections of imaginal discs and mounting slides with Fluoromount-G® mounting medium. Haltere and wing imaginal discs were isolated on glass slide, were observed under confocal microscope (see **Section 3.10**). At least thirty imaginal discs were analyzed from two separate crosses to observe effect on immunostaining of Ubx, H3K4me3 and H3K27ac.

3.4.3 Immunostaining of polytenes

Third instar larvae used for immunostaining of polytene chromosomes should be healthy so the flies used for laying eggs were kept at 18^{o}C and also fresh yeast paste was added to the vials containing the eggs. Once the eggs develop into third instar larvae, dissection of the larvae for isolation of salivary glands was done on dissection slide with depression in 1X PBS. To this end, salivary glands were pulled out with the help of a forceps and imaginal discs and fat bodies were removed prior to the fixation of salivary gland. Once the salivary glands were cleaned, they were transferred on a poly-l-lysine coated slide with fixation solution (**Appendix B**). After 15-20 minutes' incubation in fixation solution, a coverslip was placed on salivary glands followed by tapping with a sharp pencil while firmly holding the coverslip. The slide with the coverslip was squashed using slide presser to flatten the polyene chromosomes and then viewed under microscope to decide if there are good quality polytenes spread. Without wasting much time, the slide was immediately placed in liquid nitrogen to flash freeze the sample. Coverslip was removed from the slide using a sharp blade with a single jolt and the slide was placed in slide jar containing 1X PBS.

The slides were washed twice with 1X PBS by rapid shaking with IKA™ KS 501 Digital Rotary Orbital Shaker at room temperature and then once with PBST (**Appendix B**). After washing, polytenes were blocked using blocking buffer (**Appendix B**) for 1-2 hours and then the slides were rinsed with PBS and dried using paper towels without touching on the side which had polytene squashes on it. Antibody dilution made in antibody buffer (**Appendix B**) was placed on the polytene squashes and a coverslip was placed gently. All the slides prepared were placed in a humid chamber and incubated overnight at 4^{o}C.

Next morning, slides were rinsed with 1X PBS again and washed with 1X PBS thrice using IKA™ KS 501 Rotary Shaker at room temperature for 5 minutes. After washing, slides were again dried and treated with secondary antibody dilution (**Appendix B**) as mentioned above. Incubation time for secondary antibody treatment was 2 hours at room temperature. Slides were again rinsed with 1X PBS and then washing with wash buffer 300 (**Appendix B**) was done for 20 minutes at room temperature on IKA™ KS 501 Rotary Shaker. It was followed by washing with wash buffer 400 (**Appendix B**) then rinsing and drying of the slides was done prior to the addition of DAPI (0.2mg/ml) to the slides for 30 minutes. Finally, Fluoromount-G® Mounting Medium was used to prepare the slides. Immunostained polytene chromosomes were observed with confocal microscope (see **Section 3.10**).

3.5 RNA isolation and cDNA synthesis

To isolate RNA, embryos were frozen with liquid nitrogen in a microcentrifuge tube and then grinded using tube pestles (on ice). The homogenized powder was dissolved completely in 500µl TRIzol® reagent (Life Sciences), and RNA was isolated according to manufacturer's instructions with slight modifications described as following. After a 2-3 minutes incubation in TRIzol at room temperature, 100µl chloroform was added and mixed by vortexing followed by incubation at room temperature for 2-3 minutes. After centrifugation (12000 rpm) for 15 minutes at 4^{o}C, aqueous layer was isolated in fresh microcentrifuge tube and isopropanol in 1:1 ratio was added. After incubation at room temperature for 10 minutes, samples were again spun for 15 minutes at 4^{o}C and 12000 rpm. The supernatant was discarded and the pallet was washed with 75% ethanol by centrifugation using same conditions as

described previously. Supernatant was again discarded and the pellet was allowed to dry at room temperature. The RNA pallet was then dissolved in RNAase free water and was used for cDNA synthesis. The same procedure was used for isolating RNA from cultured cells by harvesting cells and resuspending them in 500µl TRIzol® reagent.

cDNA synthesis was done using SuperScript™ III First-Strand Synthesis kit (Life Sciences) using recommended protocol. The experiments in which cDNA was used as template for end-point PCR, PCR master mix was prepared as described in **Appendix B**. The experiments where cDNA was used for quantitative real time PCR, the primers of each target were used in two wells of the qRT-PCR plate. Details of primers are given in **Section 3.12**. *Power*™ SYBR® Green Real-Time PCR master mix (Thermo Fisher Scientific) was used for setting up real time PCR and relative quantification was done by $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

3.6 Plasmids constructions

To clone full-length CDS of *ball*, cDNA prepared from *Drosophila* embryos was used as the template. For cDNA preparation, total RNA from *Drosophila* embryos was isolated by above mentioned TRIzol™ RNA extraction method. Once the cDNA was prepared, it was used as template in PCR reaction using *ball* gene specific primers to amplify *ball* CDS. Amplified *ball* CDS was purified by PureLink™ Quick Gel Extraction kit (Thermo Fisher Scientific) using manufacturer's protocol and cloned in pENTR™D-TOPO® entry vector (Thermo Fisher Scientific) using the manufacturer's instructions. To confirm the successful cloning, plasmid DNA was isolated using PureLink™ Quick Plasmid prep kit according to the manufacturer's instructions and subsequently restricted as described in **Appendix B**.

Once the *pENTR™D-TOPO®-ball* construct was confirmed, Gateway ®LR cloning method (Thermo Fisher Scientific) was used to generate *ball* CDS containing two destination vectors taken from DGVC (*Drosophila* Gateway Vector Collection). First destination vector was *pMTWHF* in which the aim was to clone *ball* CDS in-frame with *8x His* and *3x FLAG* epitope tags under copper inducible *metallothionein* promoter. The second destination vector was *pAWG* in which the aim was to clone *ball* CDS in-frame with *EGFP* tag under *Actin5C* promoter. LR Clonase reactions

(Thermo Fisher Scientific) were set between *pENTR™D-TOPO®-ball* and each of the destination vectors according to manufacturer's instructions to generate the desired *pA-ball-G* and *pMT-ball-HF* plasmid constructs. To confirm the successful cloning, restriction digestion of the plasmids was done as described in **Appendix B**.

3.7 Transfection of S2 cells with *ball* constructs

The transfection was done in six well plate with Effecten® transfection reagent (Qiagen) according to manufacturer's guidelines. The EGFP tagged *ball* construct (*pA-ball-G*) was transiently transfected while the inducible FLAG-tagged *ball* construct (*pMT-ball-HF*) was used to stably transfect the *Drosophila* S2 cells. 24 hours after the transfection, the transfection media was removed from the cells to avoid cytotoxicity. Transiently transfected cells were incubated for 72 hours after transfection and before imagining. To prepare cells for imaging, cells were harvested, transferred on a coverslip and fixed by incubating in 4% formaldehyde for 30 minutes. Finally, these cells were incubated in DAPI for additional 30 minutes and the coverslips with cells were transferred to the glass slides. Details of microscopy are given in **Section 3.10**.

For stably transfecting the cells with *pMT-ball-HF* construct, the Effecten® transfection reagent manufacturer's guidelines were followed. The selection procedure and passaging of the cells was done according to the '*Drosophila* Schneider 2 (S2) Cells user guide' (Catalog Number R690-07). As the *pMT-ball-HF* construct contains the Hygromycin-B resistance gene, selection was done with media containing 300µg/ml Hygromycin-B. Induction of cells was done with 0.5mM $CuSO_4$ and expression of FLAG-tagged Ball protein was confirmed after 72 hours from induction by western blotting. Details of western blotting procedure are given in **Section 3.9**.

3.8 Chromatin immunoprecipitation

During this study, chromatin immunoprecipitation was performed from different sources that include S2 cells, stable cell lines expressing FLAG-tagged Ball or FLAG tag alone and D.Mel-2 cells treated with dsRNA against *LacZ* or *ball*. The starting material for ChIP from S2 cells and stable cell lines was three 80-95% confluent T75

flasks but for ChIP from dsRNA treated cells, three wells of a 6-well plate fully confluent with cells were used. Therefore, ChIP from dsRNA treated cells was essentially a scaled-down procedure of ChIP from S2 or stable cell lines and all relevant information is given under the term scaled-down procedure wherever deemed necessary. All the solutions and dilutions, relevant to ChIP procedure are given in **Appendix C**.

Cells for ChIP were harvested in a canonical flask with continuous gentle stirring using a magnetic stirrer. The fixative solution was added to the cells in such a manner that the final concertation of the fixative solution is 1X and that of formaldehyde is 1%. After adding fixative solution to the cells, strictly 10 minutes were given to avoid over fixation. To stop fixation, 2.5M glycine was added such that final concentration of glycine was 0.125M in the solution. Next, these fixed cells were collected by centrifugation at 2000 rpm for 5 minutes at 4^{o}C. The harvested cell pellet was further washed with 1X PBS to remove glycine and fixative solution remnants. Afterward, the cells were lysed by resuspending them in 5ml Solution A (2ml Solution A in scaled-down ChIP procedure). The re-suspended cells were incubated on ice for 5 minutes and then pelleted down by centrifugation at 2000 rpm for 5 minutes on 4^{o}C. The supernatant was discarded and pellet was re-suspended twice in 5ml of Solution B (2ml Solution B in scaled-down ChIP procedure). It was also followed by incubation on ice for five minutes and subsequent centrifugation at 2000 rpm for additional five minutes on 4^{o}C. The supernatant was discarded and the pellet was dissolved in sonication buffer containing protease inhibitors. Volume of sonication buffer was calculated on the basis of cell count of the starting material. Every 3×10^{7} cells (1×10^{7} cells in scaled-down ChIP procedure) were dissolved per 320µl of sonication buffer. Aliquots of 320µl cell suspension were sonicated by Bioruptor (Diagenode) for 25 min (30sec on/30sec off) at the high power setting. The lysate was cleared by centrifugation at maximum speed for 10 minutes on 4^{o}C and supernatant was transferred to new microcentrifuge tubes.

To prepare a mixture of Protein A and G Dynabeads™ (Thermo Fisher Scientific) for ChIP, 25µl of each of the two types of beads were mixed together in a

microcentrifuge tube. Afterwards, the beads were washed thrice with 1X RIPA buffer and then re-suspended in 50µl 1X RIPA buffer. To avoid non-specific binding of chromatin to Protein A or G, pre-clearing was done by incubation of chromatin with 50µl beads for 1.5 hour at 25 rpm on 4^{o}C. The incubation was done on *WiseMix®* rotator. The supernatants from the microcentrifuge tubes containing chromatin and Dynabeads™ were harvested using DynaMag™-2 magnet. The supernatants were then divided into input and IP samples at this stage and the input aliquots were placed on -20^{o}C till the immunoprecipitation is complete from the IP samples. The desired antibody was added to the IP samples and incubated on *WiseMix®* rotator at 25 rpm for overnight on 4^{o}C. Details of antibodies used and relevant information is given in **Section 3.11**. On next morning, the IP samples were transferred to the microcentrifuge tubes with Dynabeads™, prepared as mentioned before. The microcentrifuge tubes were incubated for four hours on 4^{o}C while rotating at 25 rpm on rotator. After incubation, the microcentrifuge tubes were placed in DynaMag™-2 magnet to remove supernatant. The beads were washed thrice with 1X RIPA buffer, once with lithium chloride buffer and once with TE buffer. All washings were done by re-suspending beads, then rotating microcentrifuge tubes for 5 minutes at 25 rpm on rotator at 4^{o}C. All washing reagents were removed using DynaMag™-2 magnet. Once the washings were complete, the chromatin was eluted from beads with 250µl of freshly prepared elution buffer, incubating the beads at 65^{o}C for 15min with gentle agitation. The elution step was repeated twice and each time nearly 250µl supernatant was collected placing the microcentrifuge tubes in DynaMag™-2 magnet. The next step was reverse cross-linking the chromatin samples together with input samples saved earlier. Chromatin was reverse cross-linked by adding 20µl of 5M NaCl and incubating at 65^{o}C overnight. Next day, each sample was treated with 10µl of 0.5M EDTA (pH 8), 20µl 1M Tris-Cl (pH 6.5) and 2µl (10mg/ml) proteinase K by gentle agitation at 45^{o}C for two hours.

Next, the DNA was purified from reverse cross-linked samples using phenol-chloroform extraction method. For that purpose, equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to each of the reverse crosslinked chromatin samples. The mixture was vortexed for 30sec, followed by

centrifugation for 13000 rpm for 2min. The upper aqueous layer from each sample was transferred to a separate new microcentrifuge tube. Re-extraction was performed with 100µl 1X TE from the mixture and added to the previously collected supernatant. Chloroform equal to the volume of aqueous layer was added and mixed vigorously on vortex for a few seconds. To collect aqueous layer again, samples were spun in a centrifuge at 13000 rpm for 10 minutes at 4^{o}C. Next, 1µl glycogen, 1/10th volume of 3M sodium acetate (pH 5.2) and 2.5 volume of absolute ethanol was added to the aqueous layer and incubated at -80^{o}C overnight. Samples were spun in a centrifuge at 13000 rpm for half an hour to precipitate DNA. The supernatant was discarded and the DNA pellet was washed with 70% ethanol. After washing, the pellets were air-dried for 10 minutes at room temperature and finally dissolved in PCR-grade water. Real-time PCR was set up using the DNA such that primers of each target were used in two wells of the qRT-PCR plate. Details of primers are given in **Section 3.12**. *Power*™ SYBR® Green Real-Time PCR master mix (Thermo Fisher Scientific) was used for setting up real-time PCR and relative quantification was done by $\Delta\Delta C_t$ method (Livak and Schmittgen, 2001).

3.9 Western blotting

To perform western blot analysis, samples were resolved on 12% polyacrylamide gel. along the SeeBlue™ Plus2 Pre-stained Protein Standard (Thermo Fisher Scientific) (details of all the solutions for western blotting are given in **Appendix D**). Electro-transfer method is used to transfer proteins on nitrocellulose membrane after briefly washing the gel with Milli-Q water twice. The nitrocellulose membrane was cut to approximately the size of 7.5×10cm and soaked in transfer buffer. In addition to membrane, the fiber pads, gel and filter papers were also equilibrated in transfer buffer for 5 minutes. Proteins were transferred on nitrocellulose membrane following standard method (Sambrook and Russell, 2001). Proteins were transferred at a constant current of 100mA at 4^{o}C for overnight. Next morning, membrane was blocked with 3-5% Non-fat milk prepared in 1X PBS for one hour. Incubation of membrane with primary antibody was performed at 4^{o}C overnight. The membrane was washed with PBST for 5 minutes before incubation with secondary antibody for 2 hours. Details of antibodies and the dilutions used in this study are given in **Section**

3.11. After incubation with secondary antibody, the membrane was washed thrice with PBST for 10 minutes each, then washed twice with 1X PBS for 5 minutes each. Before developing the blot, the membrane was rinsed with Milli-Q water. The ECL detection reagent was dropped on PARAFILM® M and the membrane was placed on it with proteins facing downwards. The amount of ECL reagent used is according to the size of membrane and usually falls between 400-900µl. After incubating with ECL reagent for 1-2 minutes, the excessive ECL was drained by touching a corner of membrane with tissue paper. The membrane was finally placed on saran wrap film and developed on ChemiDoc XRS+ Imaging System for 2 seconds to 2 minutes.

3.10 Microscopy

For observing extra sex comb phenotype Olympus SZ51 stereomicroscope was used. For imaging loss of abdominal pigmentation phenotype, the male flies of desired genotype were transferred to 70% ethanol to dehydrate. The dehydrated flies were dissected under Olympus SZ51 stereomicroscope on a dissection slide. The abdominal portion of the fly body was isolated and rehydrated in water for 5-10 minutes. After rehydration, abdomens were mounted on a glass slide in Hoyer's medium (**Appendix B**). A coverslip was placed over the specimen and then the glass slide was incubated at 65^{o}C for 50 minutes. The final imaging was done using Nikon C-DSS230 epifluorescence stereomicroscope at 3.5X magnification. The same epifluorescence stereomicroscope was used for the selection of GFP negative embryos (homozygous *ball*^{2} mutant embryos) in experiments where immunostaining and real-time PCR analysis of embryos was performed. Imaging of embryos was done at 20X magnification on Nikon C2 confocal microscope. NIS elements image acquisition software was utilized during confocal microscopy.

All larval dissections for polytene chromosomes and imaginal discs immunostainings were performed using LABOMED CZM6 stereo zoom microscope and Nikon C2 confocal microscope was used for imaging. For imaginal discs, 20X magnification was utilized while 60X was used for visualizing polytene chromosomes. Moreover, the same microscope was also used for imaging fixed cells at 40X magnification.

3.11 Antibodies

Following antibodies were used during this study: mouse anti-Ubx (DSHB, Fp3.38, IF: 1:20), mouse anti-Abd-B (DSHB, 1A2E9, IF: 1:40), rabbit anti-Trx (gift from R. Paro, IF: 1:20), rabbit anti-Pc (Santa Cruz, D220, IF: 1:20), rabbit anti-Ball (Gift from Alf Herzig, IF 1:20, ChIP: 2µl) mouse anti-GFP (Roche, 11814460001, IF:1:50), mouse anti-Tubulin (Abcam, ab44928, WB: 1:2000), mouse anti-FLAG M2 (Sigma Aldrich, WB: 1:2000, ChIP: 5µl), mouse anti-H2A-ubi (Millipore, 05-678, ChIP: 5µl), rabbit anti-H3K27ac (Abcam, Ab4729, IF: 1:75), mouse anti- H3K4me3 (Abcam, Ab1012, IF: 1:50). HRP conjugated secondary antibodies (Abcam) were used at 1:10,000 dilution for western blotting while cy3 and Alexa Fluor® 488 conjugated secondary antibodies (Thermo Fisher Scientific) were used at 1:100 dilution.

3.12 Primers list

Primers details are compiled in two lists, the list of primers for knockdown of genes is in **Appendix F** and primers for ChIP, real-time PCR and cloning are shown in **Table 3.2**.

Table 3.2: List of primers used during this study.

Primer Name	Sequence
Primers used for ChIP	
***bxd*-s-low**	GCACTTAAAACGGCCATTACGAA
***bxd*-s-up**	GACGTGCGTAAGAGCGAGATACAG
***Dfd* F**	AACTCTCCGTGCGAGCGAAC
***Dfd* R**	ATGCTCCCTCTCAGTCGCGCT
***disco*_F**	GTTTCGTTGGGTTGACACATG
***disco*_R**	CATTGCCATTTCACTCTCGTTG
***iab7*-4-low**	AGCTTTTGCCACTCGTCCTGTT
***iab7*-4-up**	AGCAGAGCTGTGCCATTGTTT
Intergenic Region F	CCGAACATGAGACATGGAAAA
Intergenic Region R	AAAGTGCCGACAATGCAGTTA
***pnr*_TSS_F**	TCTCTTGCTCTTTCGCTCAC
***pnr*_TSS_R**	GTTTTCCATACGCACTCACAC
***pnt*_TSS_F**	TCATTCCAGCGATCAAGTAAAA
***pnt*_TSS_R**	TCTTTCTCTCCGCTGCTAAGAT
***psq*_TSS_F**	ATAAGGCGATGCCACCTAGTTA
***psq*_TSS_R**	AATGTAGCAAAAGGTGCTCAAAG
***pnr*_Gene Body_F**	CCCAGTGGCGACTCATTAGA

Continued from previous page	
***pnr*_Gene Body_R**	AGATTGTGTAGTGGTCGAGCA
***pnt*_Gene Body_F**	AGTCCATCAAGATTGTGCGC
***pnt*_Gene Body_R**	TAAAATAGCGCCCCTTCGTG
Primers used for Real-time PCR analysis	
***Act57B* F**	TGTGTGACGATGAAGTTGCTGC
***Act57B* R**	ATCACCGACGTACGAGTCCTT
***AbdA* F**	TGACGCTTACAGACTGGATGG
***AbdA* R**	CGCGCCTGTTCATTTATTTCC
***AbdB* ex1.1 F**	CAACTACCGAACTAAGCTGC
***AbdB* ex1.2 R**	CACAATGAGGAGCAAGGATG
***Dfd* F**	CGATGGCGAACGGATCATCTA
***Dfd* R**	GCGTCAGGTAGCGGTTGTAGTGG
***pnr*_E2F**	GATGGAACCGGACACTATCTGT
***pnr*_E3R**	AAAGTGTGGTGGTCCGAGTG
***pnt*_E5F**	ACCTGGACCCCTTCTACAAGAT
***pnt*_E5R**	CCGTGCACAATATCCTCATTT
***rp49* F**	GACGCTTCAAGGGACAGTATCTG
***rp49* R**	AAACGCGGTTCTGCATGAG
***Ubx* F**	ATGAACTCGTACTTTGAACAGGC
***Ubx* R**	CCAGCGAGAGAGGGAATCC
***psq*_E3F**	GCAAACATCCCACAATTATCCT
***Psq*_E4R**	TCGCAGAGTCCCTTGATCTT
Primers used for *ball* cloning	
Ball-F	CACCATGCCGCGTGTAGCCAAGC
Ball-R-WS	CTATCCCTGGTATTTCCGCACAGTGACGGC
Ball-R-NS	TCCCTGGTATTTCCGCACAGTGACGGC

4 RESULTS

4.1 Kinome-wide RNAi screen to reveal novel trxG like kinases

In order to identify the cell signaling genes that play a role in trxG driven maintenance of gene activation, a previously characterized PRE based reporter system was utilized (Umer et al., 2019). In this reporter, the *Firefly* luciferase gene is expressed under the *Ubx* promoter and minimal *bxd* PRE. Minimal *bxd-PRE* that is used in the reporter has already been characterized to maintain the functionality of full-length *bxd* (Fritsch et al., 1999). In addition, *PBX (postbithorax)* (Zhang et al., 1991) and *IDE (Imaginal Disc Enhancer)* (Christen and Bienz, 1994) enhancers are also in the reporter to enhance its robustness, thus named *PBX-bxd-IDE-F.Luc* (*PRE-F.Luc*). The sensitivity of the *PRE-F.Luc* is also well characterized by gradually increasing Polycomb concentration and analyzing *F.Luc* repression in a dose-dependent manner. The reporter has also been described to respond specifically to the knockdown of different trxG members (Umer et al., 2019).

Using this reporter, an *ex vivo* RNAi screen was performed, covering all known and predicted kinases and their associated proteins from the HD2 dsRNA library (Horn et al., 2010). *Drosophila* cells treated with dsRNAs against known and predicted kinases were transfected with *PRE-F.Luc* reporter along with *Actin5C* promoter-driven *Renilla* luciferase, used as normalization control (**Methodology Section 3.1: Figure 3.1**). Each gene was knocked down in triplicates and the entire experiment was performed twice (**Figure 4.1**). Importantly, dsRNAs against known trxG members (*trx*, *ash1*) and specific reporter gene (*F.Luc*) were used as positive controls, whereas dsRNA against *LacZ* or *GFP* was used as negative control in all plates of the screen. After five days of transfection, the activity of both *Firefly* and *Renilla* luciferase was determined and Z-scores were calculated. Based on the Z-scores obtained from positive controls (*trx, ash1*), the cut-off was defined (**Figure 4.1B**) and a list of potential trxG regulators was generated (**Table 4.1**). Out of 400+ genes that were knocked down in the screen, twenty-eight specifically resulted in the reduction of *PRE-F.Luc* expression, an effect similar to the knockdown of *trx* and *ash1*.

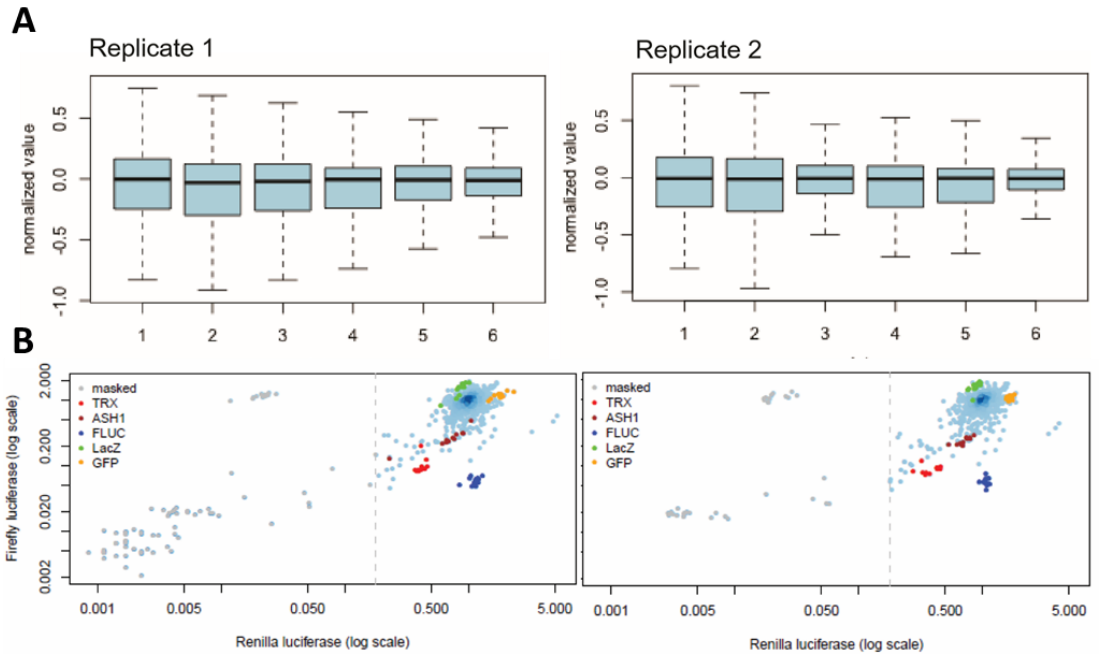

Figure 4.1: Data analysis and validation of the kinome-wide RNAi screen. (A) Median normalized data represented in box plots for all six plates of both replicates of kinome-wide RNAi screen. (B) Scatterplots of the plate median corrected intensity values for *Firefly* Luciferase (F.Luc) against the plate median corrected intensity values for *Renilla* Luciferase (R.Luc). To generate a list of trxG candidate genes that affect gene activation, Z-scores of *trx* (red) and *ash1* (maroon) knockdowns were used to set cut-off value, represented by a dashed line. Knockdown of *trx* (red) and *ash1* (maroon), as well as knockdown of *F.Luc* (blue), were used as positive controls. Cells treated with dsRNA against *LacZ* (green) or *GFP* (orange) served as negative controls. Genes affecting the activity of both F.Luc and R.Luc were masked (gray) and were not investigated further. Data shown represent two independent experiments of the kinome-wide RNAi screen.

4.2 Analysis of candidate genes using bioinformatics tools

The list of candidate genes generated from the kinome-wide RNAi screen is heterogeneous just as the trxG itself is diverse. The majority of the candidates were protein kinases but it also included regulatory subunits of kinase complexes, kinase inhibitors, nucleotide kinases and a few lipid kinases. Remarkably, the only trxG member with predicted kinase activity; *fs(1)h* was also in the list, confirming the validity of the screen. To analyze the list systematically, different tools and databases available online were exploited. A table describing known molecular function and human orthologs of each candidate gene was generated using Flybase and Uniprot (Universal protein resource) databases (**Table 4.1**) (Bairoch et al., 2005; Bateman, 2019; Thurmond et al., 2019; 1998a).

Table 4.1: List of candidate genes along with their respective Z-scores, annotation symbols, human orthologs and a summary of their known function. General descriptions of each gene were obtained from Uniprot and Flybase databases.

Candidate genes	Z-score	Annotation Symbol	Summary of known functions/General Description	Human Orthologs
Cyclin-dependent kinase 12	16.45	CG7597	Hyperphosphorylates the C- terminal heptapeptide domain of RNA Pol-II	*CDK12, CDK13*
Sphingosine kinase 1	15.27	CG1747	Converts sphingosine to sphingosine 1-phosphate	*SPHK1, SPHK2*
CG13369	13.17	CG13369	Ribokinase	*RBKS*
female sterile (1) homeotic	10.72	CG2252	Involved in pattern formation by regulating homeotic genes expression	*BRD2, BRD3, BRD4, BRDT*
S6 Kinase Like	10.62	CG7001	Promotes proteasomal degradation of BMP receptor tkv thus inhibits BMP signaling	*RSKR*
polo	9.75	CG12306	Control different aspects of cell division	*PLK1, PLK2, PLK3*
abnormal wing discs	9.7	CG2210	A nucleotide diphosphate kinase, that is involved in the biosynthesis of nucleotide triphosphates	*NME1, NME1-NME2, NME2, NME3, NME4*
dacapo	8.49	CG1772	CDK inhibitor from the CIP/KIP family that inhibits the CycE-CDK2 complex	*CDKN1C*
crossveinless 2	8.48	CG15671	A secreted protein that can bind BMPs and their receptor tkv to either inhibit or promote BMP signaling	*BMPER*
Cyclin-dependent kinase 1	8.08	CG5363	Regulates cell cycle progression by phosphorylating hundreds of target proteins	*CDK1*
Death-associated protein kinase-related	8.06	CG32666	Involved in the development of epithelial tissues	*STK17B, STK17A*
Bub1-related kinase	7.93	CG7838	Important for spindle assembly checkpoint during the cell cycle	*BUB1, BUB1B*
aurora B	7.39	CG6620	Known to phosphorylate Histone H3 at serine 10. Cell cycle-related roles include chromosome condensation, kinetochore assembly and cytokinesis	*AURKB, AURKC*
Adenylyl cyclase 78C	7.37	CG10564	Catalyzes the synthesis of 3',5'-cyclic AMP from adenosine triphosphate in response to G-protein coupled receptor signaling	*ADCY8*
Not1	7.32	CG34407	Poly(A)-specific ribonuclease that is involved in mRNA degradation	*CNOT1*

Continued from previous page				
Salt-inducible kinase 2	7.17	CG4290	Important for lipid storage and energy homeostasis	*SIK2, SIK1B, SIK1*
Nipped-A	7.14	CG33554	Member of a chromatin-remodeling complex Tip60	*TRRAP*
Cyclin-dependent kinase 2	7.13	CG10498	Catalytic subunit of CycE-Cdk2 complex that acts during progression from G1 and S phase of mitosis	*CDK2, CDK3*
Cyclin E	6.96	CG3938	A member of the cyclin group of proteins that act as a regulatory subunit of CycE-Cdk2 complex	*CCNE1, CCNE2*
Cdk5 activator-like protein	6.63	CG5387	Regulatory subunit of Cdk5-Cdk5α complex that is active in neurons	*CDK5R1, CDK5R2*
TBP-associated factor 1	6.55	CG17603	Binds with initiator elements at transcription start sites. Part of TFIID, an evolutionary conserved multimeric protein complex involved in general transcription	*TAF1, TAF1L*
Downstream of raf1	6.55	CG15793	Dual specificity kinase that acts as MAPKK. It is activated by Raf	*MAP2K1, MAP2K2*
Arginine kinase	6.51	CG32031	Belongs to the ATP: guanidino phosphotransferase family	*CKM, CKB, CKMT1A, CKMT2*
ballchen	6.44	CG6386	A nucleosomal histone kinase that phosphorylates Histone H2A at threonine 119	*VRK1, VRK2, VRK3*
Cyclin-dependent kinase 4	6.32	CG5072	Essential for cell cycle progression and promotes cellular growth	*CDK6, CDK4*
wishful thinking	6.28	CG10776	BMP type II receptor that regulates neurotransmission at the neuromuscular junction and synaptic homeostasis	*BMPR2, AMHR2*
Homeodomain interacting protein kinase	6.27	CG17090	A member of the DYRK family of kinases that contributes to several different signaling pathways including Wingless, Notch, Hippo, JNK and cell death	*HIPK1, HIPK2, HIPK3, HIPK4*
skittles	6.15	CG9985	Converts phosphatidylinositol 4-Phosphate (PIP) into phosphoinositol-4,5-bisphosphate(PIP4,5)	*PIP5K1A*

Flybase is the database that covers information about *Drosophila* genes and genome, while the Uniprot database addresses protein sequences and annotations. In order to further classify the candidates according to their protein family, subcellular localization and molecular function, the PANTHER (Protein ANalysis THrough Evolutionary Relationships) classification system was used. As expected for a kinome-wide screen, most of the candidates were classified as transferases because kinases belong to a subfamily of transferase enzymes. Other classes of candidates that PANTHER generated according to protein families and subfamilies were; enzyme modulators, receptors, transcription factors and nucleic acid-binding proteins. Interestingly, when the candidates were categorized according to subcellular localization, PANTHER classified the majority to localize in organelle as most of the candidates reside in the nucleus. Nuclear localization of most candidates further strengthens the reliability of the screen as they may interact with other transcriptional cellular memory factors that exist in nucleus. As for classification on the basis of molecular function, the bulk of the candidates were categorized for having catalytic activity, some were categorized for signaling receptor activity. Although many candidates from our list are known to be regulators of kinases, PANTHER could only categorize one candidate for enzyme regulator activity, exposing its limitations for analyzing such data (**Figure 4.2**).

To explore how different candidate genes are linked with one another, a protein interaction map was generated using the STRING (Search Tool for the Retrieval of INteracting Genes/proteins) database. This database is used to analyze known and predicted protein-protein interactions. Both direct and indirect interactions are included on the basis of reported associations in primary databases, transfer of information between organisms and computational predictions. Analysis of all the candidate genes from kinome-wide screen by the STRING generated one single cluster of proteins and this cluster is linked with cell cycle regulation (**Figure 4.3**). Epigenetic inheritance of gene expression profiles of a parent cell to daughter cells is the fundamental attribute of transcriptional cell memory, thus the involvement of many candidates in the cell cycle was not a surprise. Rather it was encouraging as understanding roles of these candidate genes linked to transcriptional cell memory

may discover much needed information about how epigenetic states are inherited through cell cycle. One of the primary objectives of this research project was to link cellular pathways to cell memory regulation. So the candidate genes' list was analyzed with respect to pathways inside the cell. To this end, Reactome, a manually curated and peer-reviewed database for pathways was utilized, that linked the candidate genes with the cellular pathways. Again, most of the proteins were found to be linked with different aspects of the cell cycle (**Table 4.2**). Other cellular pathways that had the most number of candidate genes were; signal transduction, generic transcription pathway, transcriptional regulation by P53 and metabolism. The involvement of candidates in signal transduction and transcription indicates the success of the kinome-wide screen in achieving its primary purpose.

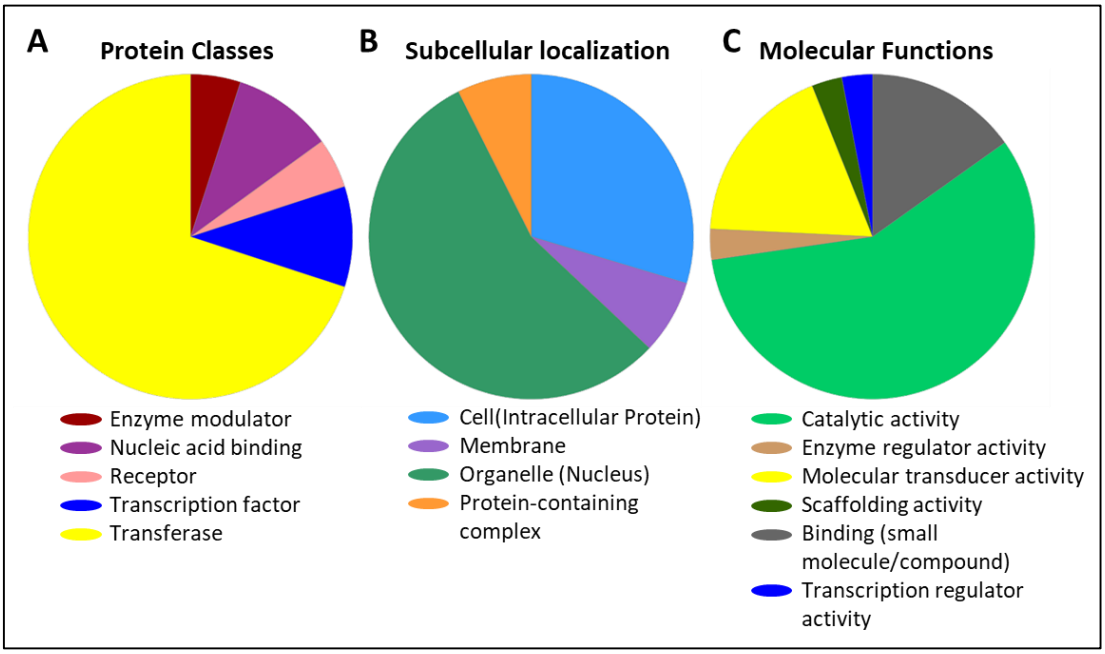

Figure 4.2: Classification of candidate proteins according to protein classes, subcellular localization and molecular function. The PANTHER classification system is utilized to generate pie charts depicting the categorization of candidates based on protein classes (A), subcellular localization (B) and molecular function (C). All the terms of different classes are from PANTHER. (A) The largest category in protein classes was transferases as kinases belong to this family. (B) Here, cell means the proteins are intracellular while all nuclear localizing proteins are under the term organelle and protein-containing complex is the umbrella term that PANTHER used for deubiquitination module complex (DUBm), catalytic complex, microtubule-associated complex and receptor complex. Nuclear localized proteins comprise the largest category in subcellular localization classification. (C) In classification according to molecular function, the majority of the candidates belonged to catalytic activity class because of their kinase activity. Here, binding is an umbrella term that PANTHER used for heterocyclic compound binding, protein binding and small molecule binding (Mi et al., 2019; Thomas et al., 2003).

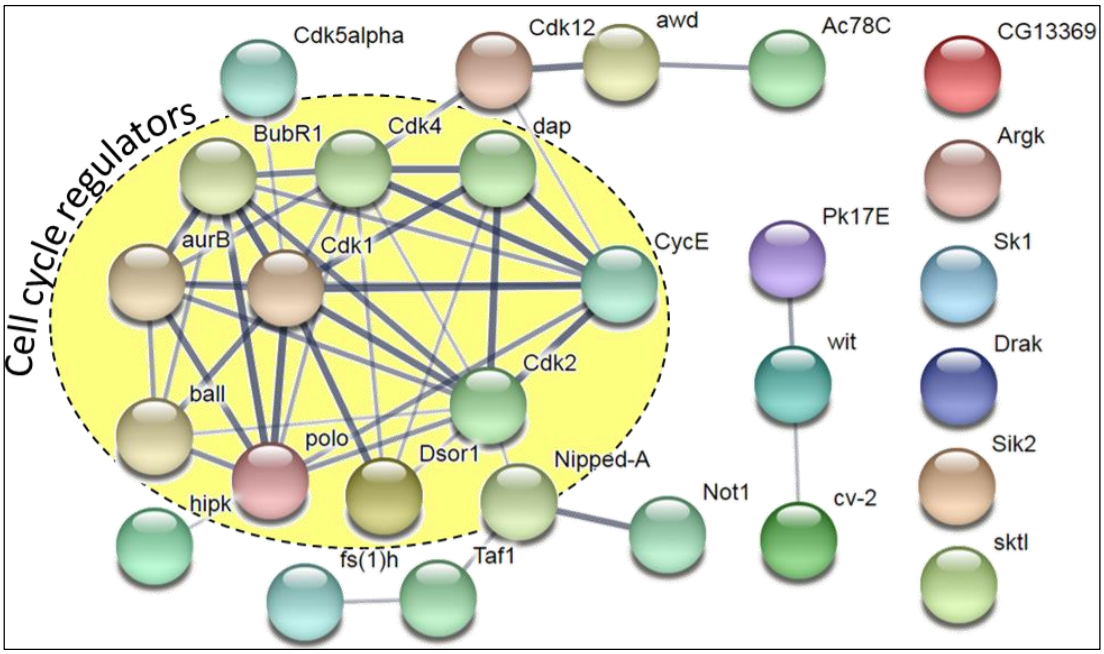

Figure 4.3: Protein-protein interaction network of candidates identified in the kinome-wide RNAi screen. A protein interaction map was generated using the STRING database. Nodes depicting proteins that are connected by lines of varying thickness. Thickness of lines demonstrates the degree of confidence for the interaction between connected nodes. The yellow circle is marking candidates that are known to associate with cell cycle regulation (Snel, 2000; Szklarczyk et al., 2019).

Table 4.2: Candidate genes linked with cellular pathways, generated using Reactome database (Fabregat et al., 2018; Joshi-Tope et al., 2005).

Pathway Names	# of Entities in the candidate list	# of Entities in database	Candidate names
Signal Transduction	9	810	wishful thinking, ballchen, Cyclin-dependent kinase 1, Adenylyl cyclase 78C, Sphingosine kinase 1, Nipped-A, skittles, S6 Kinase Like, Downstream of raf1
Cell Cycle	8	265	ballchen, Cyclin-dependent kinase 1, Cyclin-dependent kinase 2, Cyclin E, Cyclin-dependent kinase 4, Bub1-related kinase, aurora B, polo
Generic Transcription Pathway	8	363	Cyclin-dependent kinase 1, female sterile (1) homeotic, Cdk5 activator-like protein, Cyclin-dependent kinase 4, Homeodomain interacting protein kinase, aurora B, Cyclin-dependent kinase 12, TBP-associated factor 1
Transcriptional Regulation by P53	6	163	Cyclin-dependent kinase 1, Cdk5 activator-like protein, Homeodomain interacting protein kinase, aurora B, Cyclin-dependent kinase 12, TBP-associated factor 1
APC/C-mediated degradation of cell cycle proteins	5	84	Cyclin-dependent kinase 1, Cyclin-dependent kinase 2, Bub1-related kinase, aurora B, polo
Metabolism	5	1148	Arginine kinase, CG13369, abnormal wing discs, Sphingosine kinase 1, skittles
Immune System	4	863	ballchen, abnormal wing discs, Downstream of raf1, Cyclin-dependent kinase 12
Mitotic G1-G1/S phases, S phase	3	108	Cyclin-dependent kinase 2, Cyclin E, Cyclin-dependent kinase 4
Cell Cycle Checkpoints	3	122	Cyclin-dependent kinase 1, Cyclin-dependent kinase 2, Bub1-related kinase
Developmental Biology	3	125	Cyclin-dependent kinase 2, Cyclin-dependent kinase 4, Downstream of raf1
M Phase	3	141	ballchen, Cyclin-dependent kinase 1, polo
Intracellular signaling by second messengers	3	164	Adenylyl cyclase 78C, skittles, S6 Kinase Like

4.3 Validation of kinome-wide RNAi screen

Kinome-wide RNAi screen generated a list of promising candidates (**Table 4.1**) but validation of these candidates was required before genetic and molecular analysis of their physiological relevance in fly development. For this reason, a secondary screen was performed on selected serine-threonine protein kinases in the list of candidate genes. In the secondary screen, the same procedure of primary screen was repeated but dsRNA amplicons targeting a different region in these candidate genes were used from the DRSC database (Ramadan et al., 2007) and not from the HD2 library. Control cells were treated with dsRNA against *LacZ*, *trithorax* and *ash1* genes. Cells treated with *trithorax* and *ash1* dsRNA served as the positive control, while cells treated with *LacZ* dsRNA served as negative control. The knockdown of nearly all of the kinases exhibited significantly lower relative F.Luc activity as compared to the cells treated with dsRNA against *LacZ* (**Figure 4.4**).

4.4 Ballchen selection for further characterization

Next, *ballchen* (*ball*) was selected from the list of candidate genes to further elucidate its genetic and molecular link with trxG because of the following reasons. Firstly, it is a known histone protein kinase, which phosphorylates a specific threonine residue in histone H2A (Aihara et al., 2004). Secondly, it is known to be involved in both cell cycle (Fiona Cullen et al., 2005) and signal transduction pathways (Herzig et al., 2014; Yakulov et al., 2014) and is associated with chromatin condensation during meiosis (Lancaster et al., 2007), making it a suitable representative gene of the candidate list. Moreover, it has affected the reporter gene associated, relative F.Luc activity more than most of the candidates in the secondary screen.

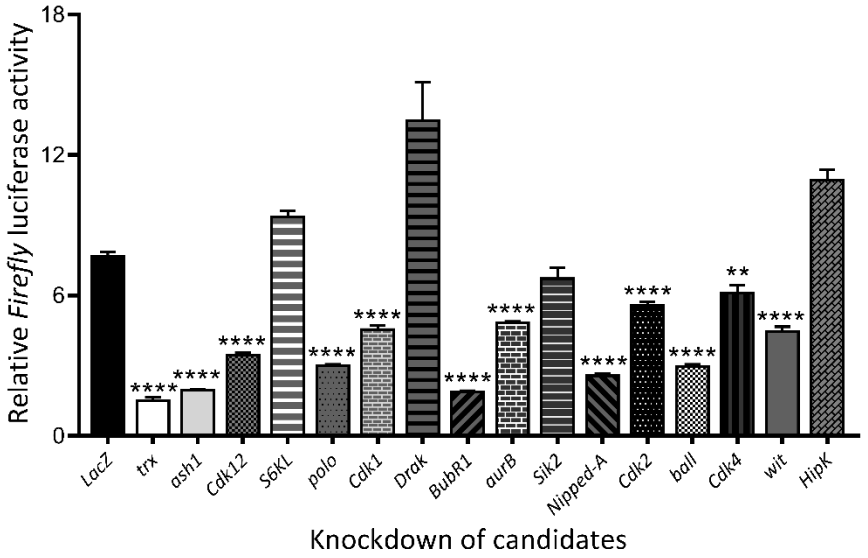

Figure 4.4: Secondary screen validated the kinome-wide RNAi screen results. D.Mel-2 cells treated with dsRNA against candidate genes or controls were transiently transfected with *PRE-F.Luc* and *pActin-R.Luc*. *Firefly* luciferase activity along with *Renilla* luciferase activity was measured after 5 days of transfection. Cells treated with dsRNA against the majority of candidate genes exhibited significantly lower relative *Firefly* luciferase activity when compared to cells treated with dsRNA against *LacZ*. R.Luc was used as internal normalization control, while cells treated with dsRNA against *trx* and *ash1* were used as positive control. The experiment was performed in triplicate and independent t-tests were done for analysis (** $p \leq 0.01$ or **** $p \leq 0.0001$).

4.5 Genetic interaction between Ballchen and PcG/trxG

4.5.1 *ball* genetically interacts with *Polycomb* (*Pc*)

Extra sex combs on second and third pair of legs in male *Drosophila melanogaster,* is a phenotype associated with PcG mutations. *Polycomb* (*Pc*) heterozygous mutants also exhibit this phenotype (Lewis, 1978). *Scr* (*Sex combs reduced*), a target of PcG/trxG, is responsible for the appearance of sex combs in wild-type male flies but normally it is expressed in the first pair of legs (Sato et al., 1985; Wakimoto and Kaufman, 1981). *Pc* is responsible for maintaining the silent state of *Scr* in the second and third pair of legs, while trxG is responsible for maintaining the active state of *Scr* in the first pair of legs (Lewis, 1978). If *Pc* is mutated in a fly, extra sex combs appear on second and third pairs of legs due to ectopic expression of *Scr* but a mutation in *trithorax* group genes suppress this *Pc* associated phenotype (Kassis et al., 2017).

To investigate if the *ball* indeed genetically interacts with the *Pc*, *ball* mutant ($ball^2$) flies were crossed to two different alleles of *Pc* (*1*, *XL5*). *Pc* heterozygous mutant males from a cross between *Pc* mutant flies and w^{1118} flies were used as control.

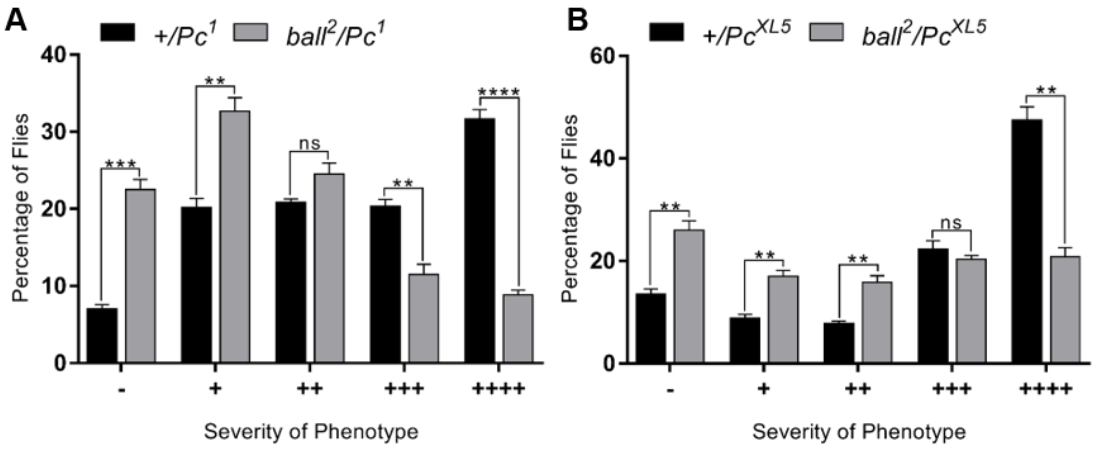

Figure 4.5: Mutation in *ball* suppresses extra sex comb phenotype of *Pc* mutations. *ball* mutant flies (*ball*^{2}) were crossed to two different alleles of *Pc*, *Pc*^{1} (A) and *Pc*^{XL5} (B), while control crosses were between *Pc* alleles (*Pc*^{1} and *Pc*^{XL5}) and *w*^{1118} flies. *Pc*^{1}/+ flies in (A) and *Pc*^{XL5}/+ flies in (B) from control crosses exhibit strong extra sex comb phenotype. In contrast, *ball*^{2} strongly suppressed both the *Pc* alleles to none or few extra sex comb in *ball*^{2}/*Pc*^{1} (A) as well as in *ball*^{2}/*Pc*^{XL5} (B). 200 male flies were analyzed for each cross and data shown represents three independent experiments. Based on the strength of phenotype, male flies were categorized according to the severity of the extra sex comb phenotype. These categories are: -, no extra sex comb; +, 1-2 hairs on second leg; ++, more than 3 hairs on second leg; +++, more than 3 hairs on second leg and 1-2 hairs on third leg; ++++, strong sex combs on both second and third pairs of legs. All crosses were done in triplicates and independent t-tests were performed for analyzing each category (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ or **** $p \leq 0.0001$).

Pc^{1}*/+* and *Pc*^{XL5}*/+* male mutants of control crosses showed a strong extra sex comb phenotype. Importantly, extra sex comb phenotype was significantly suppressed in both *ball*^{2}*/ Pc*^{1} and *ball*^{2}*/ Pc*^{XL5} double mutant flies (**Figure 4.5**) when compared with the control flies. This clearly supports the role of *ball* as a trxG-like factor and its genetic interaction with *Pc*. The categories made to score the severity of extra sex comb phenotype and the details of all the alleles used in crosses are given in **Methodology Section 3.3**: **Table 3.1** and **Appendix G: Table 7.1**, respectively.

4.5.2 *ball* genetically interacts with *trithorax* (*trx*)

In *D. melanogaster* male flies, a *Hox* gene *Abd-B* is responsible for pigmentation in A5 and A6 abdominal segments (Jeong et al., 2006). A gene named *bric-a-brac* (*bab*) suppresses pigmentation in female A5 and A6 abdominal segments but itself is suppressed by Abd-B in males (Kopp et al., 2000). It has been described that the *Hox* genes expression pattern is maintained by PcG/trxG system (Lewis, 1978). Being a *Hox* gene from the Bithorax complex (BX-C), Abd-B is expressed progressively from

A5 to A7 of the pupae due to the action of trxG and kept silent elsewhere by the action of PcG (Delorenzi and Bienz, 1990). If *trx* is mutated, Abd-B is restricted towards the posterior part of the body (Breen and Harte, 1993) thus causing loss of pigmentation in A5 of male flies (Jeong et al., 2006). This loss of pigmentation results in A5 to A4 transformation as A5 starts exhibiting the A4 segment identity. A5 to A4 transformation is enhanced if more than one member of the *trxG* is mutated in the same fly. Since *ball* mutants exhibit trxG like behavior by strongly suppressing extra sex comb phenotype, genetic interaction between *ball* and *trx* was investigated using enhanced loss of pigmentation phenotype (A5 to A4 transformation) as a readout (**Figure 4.6**).

To this end, $ball^{2}$ mutant was crossed with two different *trx* mutants (trx^{1} and trx^{E2}) and 200 flies in progeny having both *ball* and *trx* mutant alleles were scored for loss of pigmentation phenotype from each cross. Both the alleles of *trx* crossed with w^{1118} served as a control and *trx*/+ heterozygous flies in progeny were used to score A5 to A4 transformation. When compared with trx^{E2}/+ and trx^{1}/+ control flies, $ball^{2}$/*trx* trans-heterozygotes exhibited significantly more frequent A5 to A4 transformations (**Figure 4.6**). Thus revealing strong enhancement of *trx* phenotype by *ball* mutation and providing support to hypothesis that Ball is working synergistically with trxG in the cellular context.

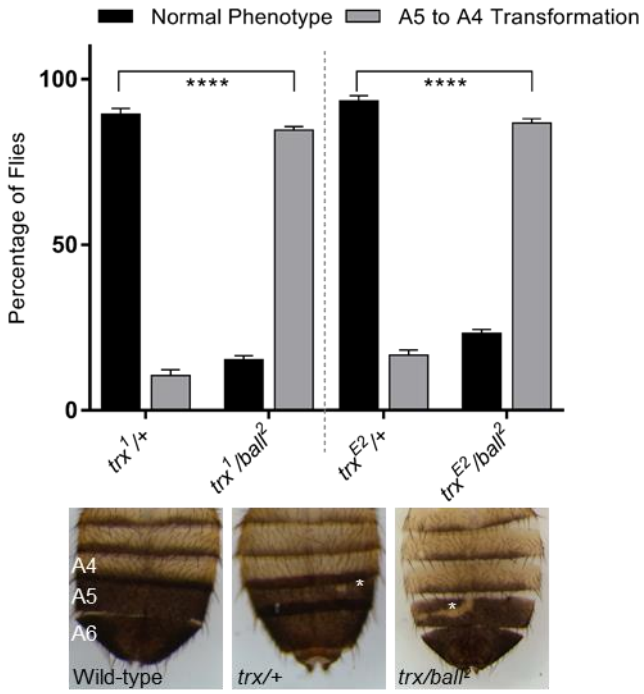

Figure 4.6: *ball* genetically interacts with *trx* and exhibits strong enhancement of A5-A4 transformation phenotype. *ball*^{2} mutant flies were crossed to two different alleles (*trx*^{1} and *trx*^{E2}) of *trx*, and males in F1 were scored for the loss of pigmentation in the A5 abdominal segment (A5 to A4 transformation, marked with an asterisk). Heterozygous *trx* mutant flies (*trx*^{1}/+ and *trx*^{E2}/+) show A5 to A4 transformation when compared to wild-type flies. Strong enhancement of A5 to A4 transformation can be seen in *ball*^{2}/*trx* double mutants when compared to *trx*/+ (top panel). Representative images for A5 to A4 transformation (marked by white asterisk) are presented (bottom). All crosses were performed in triplicates and independent t-tests were used for analyzing each category (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ or **** $p \leq 0.0001$).

4.6 Depletion of Ball affects expression of trxG targets

4.6.1 Knockdown of the *ball* alters trxG targets in *Drosophila* cells

After validating the effect of *ball* knockdown on *PRE-F.Luc* reporter multiple times, it was investigated if knockdown of *ball* affects gene expression of endogenous trxG targets in *Drosophila* cells. Towards this end, mRNA levels of transcriptionally active targets (*pnt*, *pnr*) of PcG/trxG were analyzed through qRT-PCR in D.Mel-2 cells treated with either dsRNA against *ball* or *LacZ* (**Figure 4.7**). Depletion of Ball was confirmed by western blot analysis of dsRNA treated cells with anti-Ball antibody. The expression of both *pnt* and *pnr* was significantly decreased in Ball depleted cells when compared to *LacZ* dsRNA treated cells. This substantiates the initial results that Ball indeed interacts with trxG to maintain gene expression of endogenous targets.

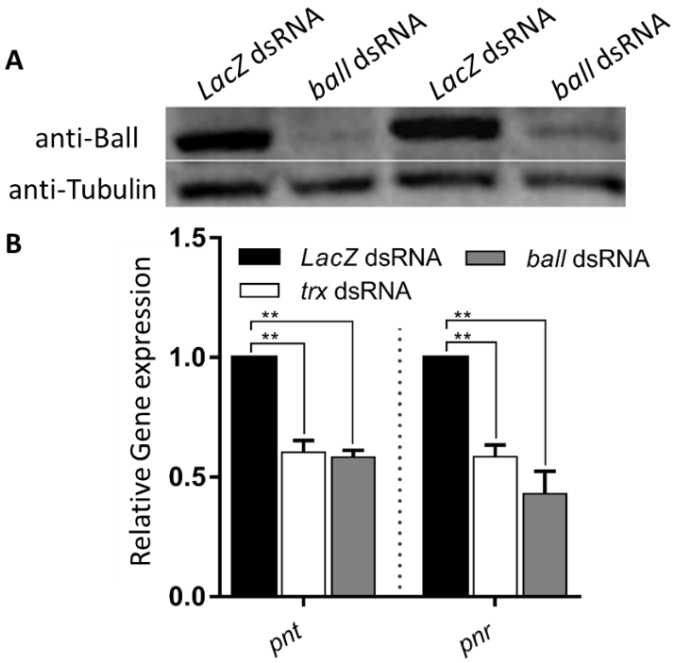

Figure 4.7: Knockdown of *ball* affected trxG target genes. (A) Depletion of Ball protein was confirmed by western blot analysis using cell lysate prepared from *LacZ* dsRNA and *ball* dsRNA treated cells, while Tubulin was used as loading control. (B) Effect of *ball* knockdown on trxG target genes (*pnt*, *pnr*) was analyzed in D.Mel-2 cells using qRT-PCR analysis. *LacZ* dsRNA treated cells were used as negative control and cells treated with dsRNA against *trx* served as positive control. Significantly lower levels of *pnt* and *pnr* were observed in cells treated with dsRNA against *ball*. Independent t-tests were performed for each gene analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ or **** $p \leq 0.0001$). The experiment was performed in triplicate.

To investigate whether depletion of Ball affects expression of trxG targets *in vivo*, the UAS-GAL4 system (see **Appendix A**) was utilized to specifically knockdown *ball* in wing and haltere imaginal discs. A *Drosophila* transgenic fly carrying *ball* inverted repeat RNAi construct under *UAS* promoter (*UAS-shRNA-ball*) was crossed with an engrailed GAL4 (*en-GAL4*) driver line that also expresses RFP under UAS enhancer. Since *en* is expressed in posterior compartment of all segments, it was anticipated that *ball* specific knockdown will coincide with RFP expression in the posterior compartments. Immunostaining of Ubx, a known target of trxG, in haltere imaginal discs was performed using third instar larvae in the progeny of *UAS-shRNA-ball* crossed with *en-GAL4* flies. This experiment was performed using two different *ball* RNAi lines to determine specificity of results. As compared to the anterior compartment (regions with no RFP signal), no observable change in Ubx expression could be visualized in haltere discs where *ball* is knocked down (**Figure 4.8**).

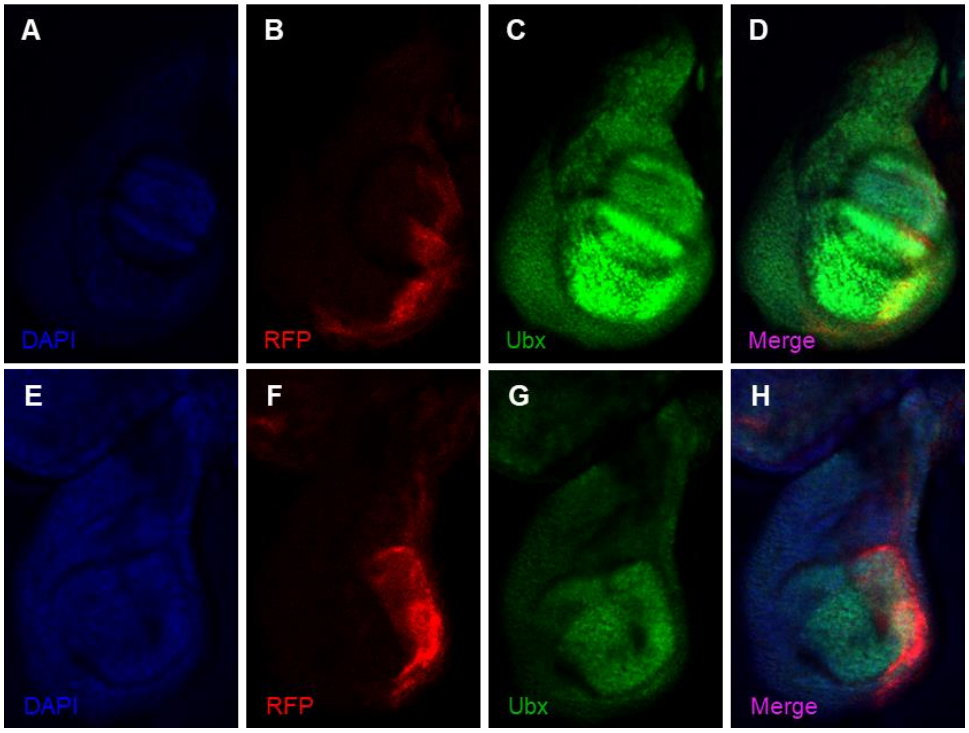

Figure 4.8: Ubx expression pattern does not alter upon knocking down *ball*. Representative images for immunostaining of Ubx in haltere imaginal discs isolated from larvae having *UAS-shRNA-ball* along with *UAS-RFP* as well as *en-GAL4*. Expression of RFP indicates posterior compartment where en-GAL4 is expressed and it indicates region of *ball* knockdown. No significant effect of *ball* knockdown upon Ubx expression was seen as expression of Ubx remains comparable in both anterior and posterior regions of the discs. Imaginal discs from two different RNAi lines (A-D) *P {TRiP.GL00068}* construct and (E-H) *P {TRiP.HMC04017}* are shown. (A, E) DAPI staining (B, F) RFP signal (C, G) immunostainings of Ubx (D, H) merge images.

It is pertinent to mention that only few mitotic clones of *trx,* where trx is completely lost, show loss of Ubx in haltere discs (Klymenko and Jürg, 2004). Therefore, failure to observe any effect of *ball* knockdown on Ubx expression in haltere discs is not that surprising. It can also be attributed to inefficiency of RNAi *in vivo* (Perrimon et al., 2010).

4.6.2 Ubx is depleted in homozygous *ball*^{2} somatic clones

Since *ball* knockdown did not show an effect on expression of Ubx *in vivo*, it was planned to generate somatic clones of *ball*^{2} in haltere imaginal discs to observe effect on Ubx expression where Ball will be completely lost. Using Flp-FRT system (see **Appendix A**), *ball* homozygous clonal cells are generated in an otherwise heterozygous mutant background. The generation of such a genetic mosaic is achieved through mitotic recombination and required producing flies that carry *ball*^{2}

allele recombined with *FRT* sites on the 3R arm of the third chromosome (*FRT3R, ball²*) near the centromere relative to the *ball* locus. These *FRT3R-ball²* male flies were crossed with females carrying FLP recombinase under the *hsp70* promoter as well as chromosome *FRT3R* linked to GFP gene under the *ubiquitin* promoter (*FRT3R, Ubi-GFP*). After 55 hours of egg-laying (48 hrs AEL), the progeny of this cross was heat shocked to generate somatic clones. Wing and haltere imaginal discs (IDs) from third instar larvae were isolated and stained with Ubx antibody.

The regions in imaginal discs (IDs) where GFP signal is absent are the somatic clones that are homozygous for *ball²* mutation. Moreover, neighboring regions with relatively high GFP signal are the twin clones which are consequence of mitotic recombination and are homozygous for wild-type *ball* as well as Ubi-GFP. This way IDs contain mosaic cells which are; homozygous for *ball* mutation, homozygous for wild-type *ball* and heterozygous for *ball²* mutation where no mitotic recombination took place. Since Ubx is normally expressed in haltere but not in wing IDs (**Figure 4.9** and **Figure 4.10**), staining with anti-Ubx provided an opportunity to examine if Ball contributes to PcG mediated silencing of Ubx in wing or trxG regulated Ubx activation in haltere IDs. In homozygous *ball²* somatic clones, marked by the absence of GFP signal, there was no effect on Ubx expression pattern in the wing IDs as it remained silent. However, Ubx expression diminished in a small subset of such clones present in the haltere IDs (**Figure 4.10**). This effect of mutation in *ball* on expression of Ubx is similar to the effect of *trx* somatic clones where such loss of Ubx is seen only in few clones in haltere discs (Klymenko and Jürg, 2004). It indicates potential role of Ball in maintenance of gene expression by trxG.

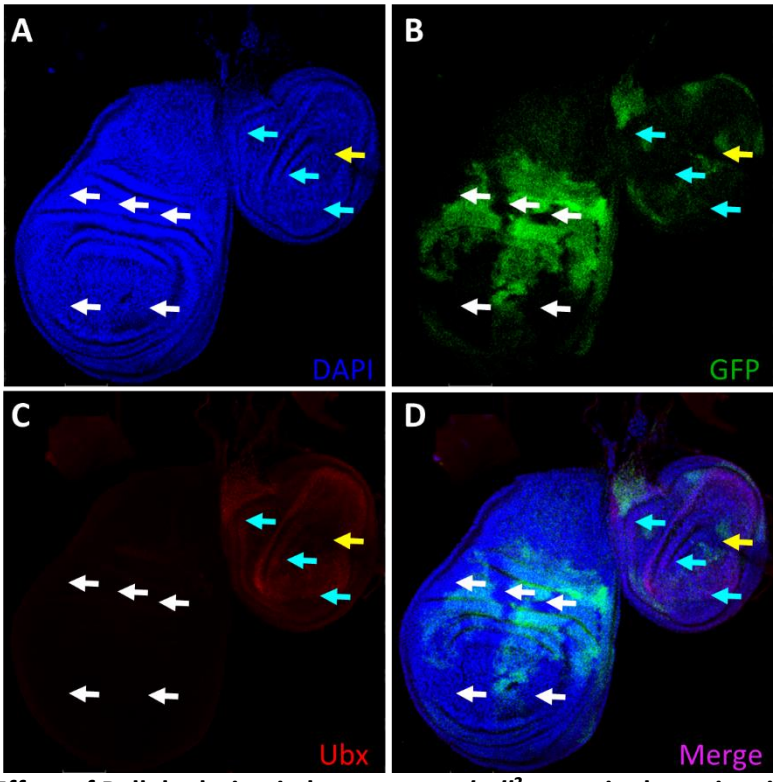

Figure 4.9: Effect of Ball depletion in homozygous *ball*^{2} somatic clones in wing and haltere imaginal discs on Ubx. A representative image for wing (left) and haltere (right) imaginal discs with mutant clones for *ball*^{2}, marked by the absence of GFP. DAPI staining (A) indicates staining of all nuclei in cells, regions lacking GFP (B) indicate *ball*^{2} homozygous clones and staining of Ubx (C) highlights effect on Ubx expression in wing and haltere discs. As Ubx is silent in wing imaginal discs, no effect on expression of Ubx in *ball*^{2} somatic clones (GFP minus regions) was seen, as no staining of Ubx is observed (white arrows). As compared to wing IDs, Ubx is expressed in haltere IDs. A majority of *ball*^{2} somatic clones have no effect on Ubx expression (cyan arrows) whereas *ball*^{2} clone marked with yellow arrow has drastically reduced Ubx expression as compared to other clones.

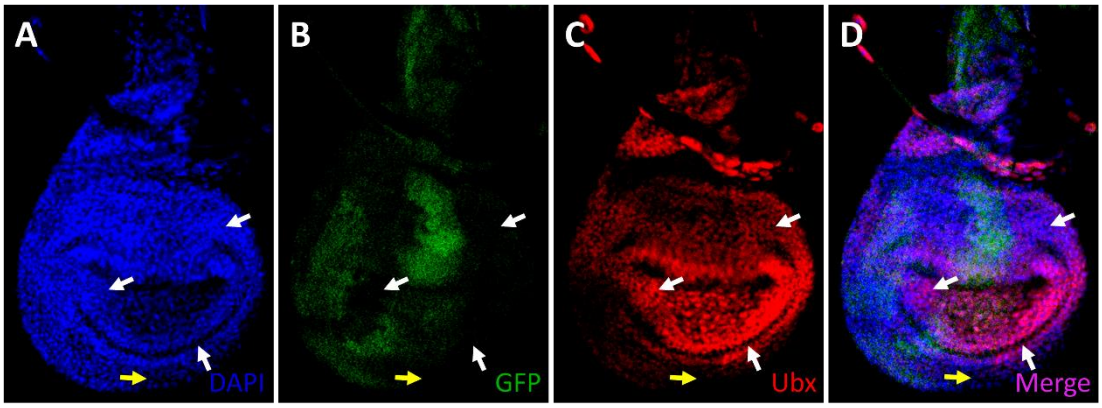

Figure 4.10: Ubx is depleted in some but not all homozygous *ball*^{2} somatic clones. A representative image of haltere imaginal disc with homozygous mutant clones for *ball*^{2}. DAPI staining (A) highlights all nuclei of the cells and the absence of GFP in (B) specifies the homozygous *ball*^{2} mutant clones. Immunostaining with anti-Ubx antibody in (C) indicates loss of Ubx in a subset of the clones (yellow arrow) but in most clones, no effect on Ubx expression was detected (white arrows). The merge image of (A), (B) and (C) is represented in (D).

4.6.3 PcG/trxG targets are misregulated in homozygous *ball²* mutant embryos

To validate effect of *ball²* on expression of trxG targets, expression of homeotic genes was analyzed in homozygous *ball²* embryos. As already discussed, *ball* is known to be highly expressed in embryos and it also explains why homozygous *ball²* embryos do not survive till adulthood. Since *ball* mutant is only viable as a heterozygote, it was necessary to positively select homozygous *ball²* mutant embryos at early embryonic stage. For this purpose, *ball²* mutation was balanced with a third chromosome balancer marked with GFP (**Methodology Section 3.3**). In the progeny of these flies, homozygous *ball²* mutant embryos were easily selected as the ones that do not express GFP. Such embryos were collected to conduct two experiments. Firstly, immunostaining of *ball²* homozygous embryos was performed with anti-Ubx and anti-Abd-B antibodies. Secondly, expression of *Abd-A*, *abd-B*, *Ubx*, *Dfd* and *pnt* was analyzed using quantitative PCR.

Both homozygous *ball²* and *w¹¹¹⁸* embryos were stained with anti-Ubx and anti-Abd-B antibodies when they are in stage 15 of development. In *w¹¹¹⁸* embryos at this stage of development, Ubx is expressed weakly in parasegment 5 (PS5), at a higher level in PS6 and then at progressively decreasing levels from PS7 to PS12 (**Figure 4.11A, B**). However, most *ball²* embryos show merged or misaligned parasegments as indicated by the Ubx staining (**Figure 4.11C, D**). In the case of Abd-B stainings, normally its expression progressively increases from PS10-14 (**Figure 4.11E, F**). However, in *ball²* mutants, Abd-B expression is drastically reduced when compared with *w¹¹¹⁸* embryos (**Figure 4.11G, H**).

Next, expression of *abd-A*, *Abd-B*, *Ubx*, *Dfd* and *pnt* was analyzed using quantitative PCR from 20-22 hours old homozygous *ball²* mutant embryos and compared with *w¹¹¹⁸* embryos. As compared to *w¹¹¹⁸* embryos, a significant reduction in expression of *abd-A, Abd-B* and *pnt* was observed (**Figure 4.12**). Reduction in Abd-B expression correlates with the diminished Abd-B signal observed in the immunostaining experiment. This result also explains the phenotypic analysis of *ball²*/*trx** trans-heterozygotes in males as pigmentation of the posterior body segments is controlled

by Abd-B (Jeong et al., 2006). Together with genetic evidence, these results indicate a close interaction of *ball* with trxG.

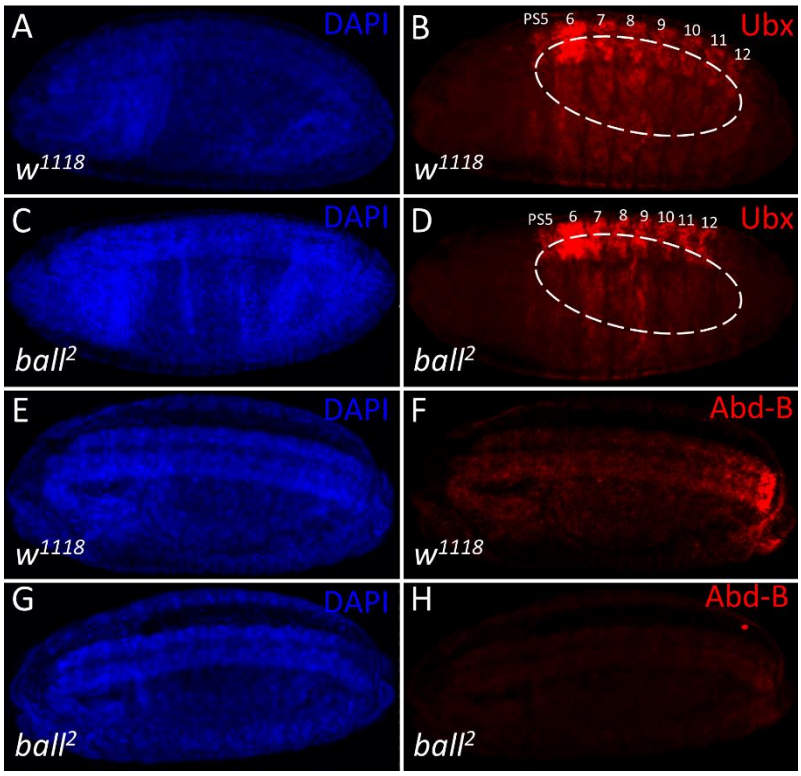

Figure 4.11: Homozygous *ball²* embryos show aberrant Ubx and Abd-B expression. Immunostainings of stage 15 embryos with anti-Ubx (A-D) and anti-Abd-B (E-H) are shown in w^{1118} (B, F) as compared to homozygous *ball²* (D, H) embryos. *ball²* embryos showed aberrant expression of both Ubx and Abd-B as compared to w^{1118} embryos.

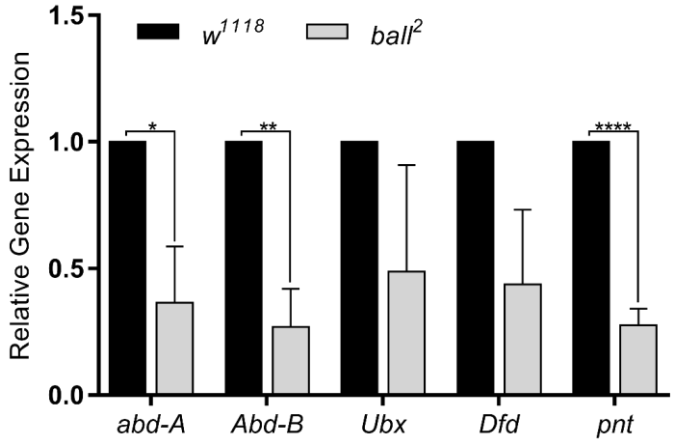

Figure 4.12: Homozygous *ball²* embryos exhibit a reduction in *abd-A*, *Abd-B* and *pnt* expression. Significantly, low level of *abd-A*, *Abd-B* and *pnt* expression was detected through qRT-PCR in homozygous *ball²* embryos when compared with w^{1118} embryos. No significant difference in levels of *Ubx* and *Dfd* was observed. Independent t-tests were performed for each gene analysis (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ or **** $p \leq 0.0001$).

4.7 H3K4me3 and H3K27ac marks are depleted in homozygous *ball*^{2} somatic clones

Since Ball is a known histone modifier and histone modifications are known to effect each other, it was questioned that whether H3K4 methylation mark mediated by COMPASS complex (Miller et al., 2001; Roguev et al., 2001) and the H3K27 acetylation mark mediated by CBP (Tie et al., 2009) are affected by absence of Ball *in vivo*. Observing the effect of Ball on trxG mediated histone marks could help to decipher the histone code underlying the gene activation and repression. The homozygous *ball*^{2} mutant clones were used to inspect the effect of *ball* mutation on H3K4me3 (**Figure 4.13**) and H3K27ac (**Figure 4.14**) marks.

The H3K4me3, a global mark for transcriptional activation (Ardehali et al., 2011; Hallson et al., 2012), was reduced in some but not all the clones but mutation in *ball* drastically affected H3K27ac. The H3K27 acetylation was significantly reduced in the majority of the homozygous *ball*^{2} mutant clones which is the histone modification more specific to trxG mediated gene activation (Tie et al., 2009, 2014).

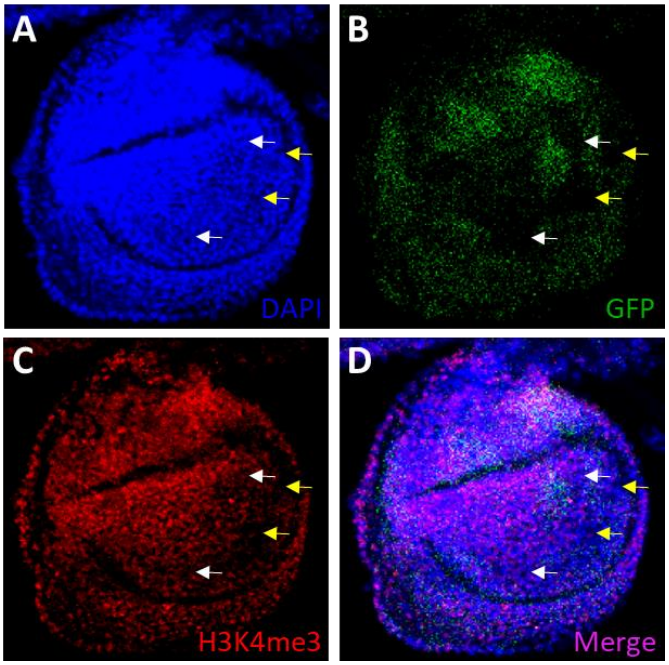

Figure 4.13: H3K4me3 is affected in homozygous *ball*^{2} somatic clones. A representative image for haltere imaginal disc with homozygous mutant clones for *ball*^{2}, marked by the absence of GFP signal. DAPI staining (A) of the haltere shows nuclei of all the cells and the absence of GFP (B) highlights the homozygous *ball*^{2} mutant clones. Immunostaining with anti-H3K4me3 antibody (C) indicates loss of H3K4me3 histone mark in some of the clones (yellow arrow) but such loss is not evident in other clones (white arrows). The merge image of (A), (B) and (C) is represented in (D).

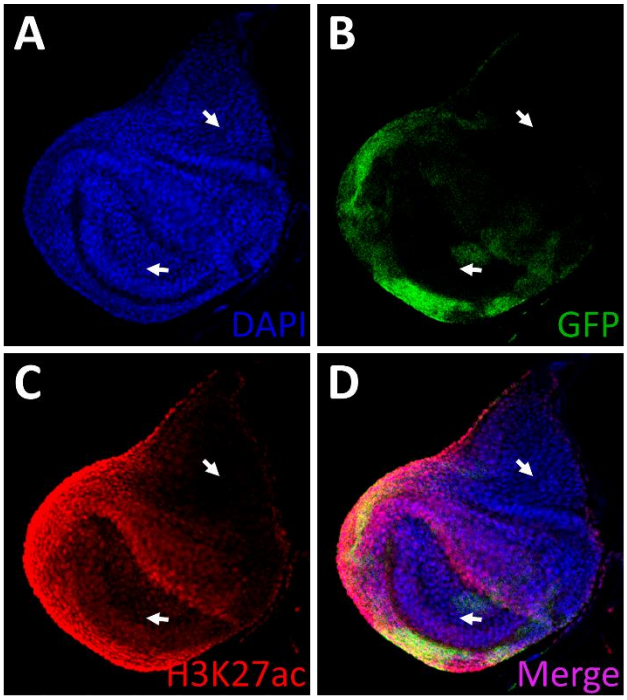

Figure 4.14: H3K27ac is depleted in homozygous *ball*^{2} somatic clones. A representative image of haltere imaginal disc with homozygous mutant clones for *ball*^{2}, marked by the absence of GFP signal. DAPI staining (A) highlights all the nuclei of the cells and the regions with no GFP signal (B) are homozygous *ball*^{2} mutant clones. Immunostaining with anti-H3K27ac antibody (C) indicates loss of H3K27ac histone mark in the *ball*^{2} mutant clones (arrows). The merge image of (A), (B) and (C) is represented in (D) which indicates overlap of GFP and H3K27ac staining in majority regions of the imaginal disc.

4.8 Subcellular localization of Ball

For molecular characterization of Ball association with trxG, it was imperative to clone full-length *ball* CDS (coding sequence). To this end, the *ball* CDS was PCR amplified from cDNA prepared from *Drosophila* embryos (**Methodology Section 3.5**). Amplified *ball* CDS was cloned in Gateway entry vector (**Figure 4.15**) and the recombinant construct containing *ball* CDS was confirmed by restriction digestion analysis with BglII (**Figure 4.15C**). BglII has two restriction sites within the CDS of *ball* that produce fragment of 844bp upon restriction digestion. The confirmed recombinant plasmid was further validated by DNA sequencing (**Appendix E**). Comparison of the DNA sequence obtained, with the *ball* CDS sequence available on Flybase database, revealed that cloned *ball* CDS has 18 bases different from the Flybase reference sequence. However, 12 of these sites are silent mutations as they do not affect amino acid sequence and the remaining 6 changes are already reported as single nucleotide polymorphs in Ensembl database (Hubbard, 2002).

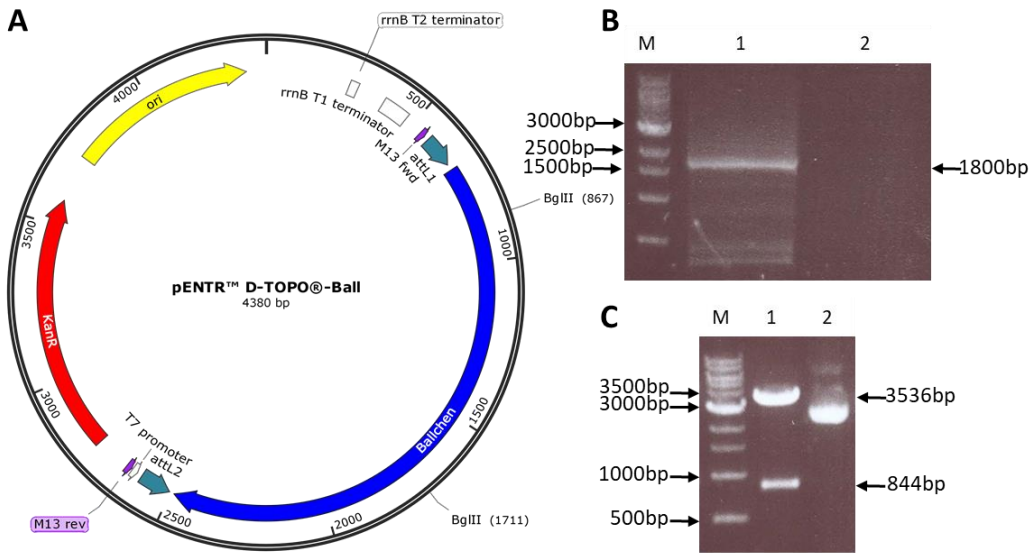

Figure 4.15: Molecular cloning of *ball* in pENTR™D-TOPO and confirmation by restriction digestion analysis. (A) Schematics of *ball* coding sequence (CDS) cloned in Gateway entry vector (*pENTR™D-TOPO®*). (B) PCR amplification of *ball* CDS (lane 1) along with negative control in PCR (lane 2) resolved on agarose gel. (C) Restriction digestion with BglII led to the release of two expected fragments of 3536bp and 844bp size that confirmed successful cloning. The restricted plasmid (lane 1) was resolved along with unrestricted plasmid (lane 2) and 1kb DNA marker (M).

The *ball* CDS was then cloned in-frame with EGFP to visualize subcellular localization of Ball (**Figure 4.16**). For this purpose, *ball* CDS entry clone was recombined with expression vector in which recombination cassette is in-frame with EGFP tag and under the control of constitutively active *Actin5C* promoter. Successful cloning was confirmed by restriction digestion with BglII which released three bands of 4541bp, 2674bp and 844bp sizes, as expected. Transient transfection of Actin-*ball-EGFP* in *Drosophila* S2 cells revealed that although Ball is solely present in the nucleus in some cells, it also existed in both nucleus and cytosol in some cells (**Figure 4.17**). Such localization pattern was expected as previously Ball is known to be present in both cytosol and nuclei in the early S phase but is concentrated on chromatin during mitosis (Aihara et al., 2004).

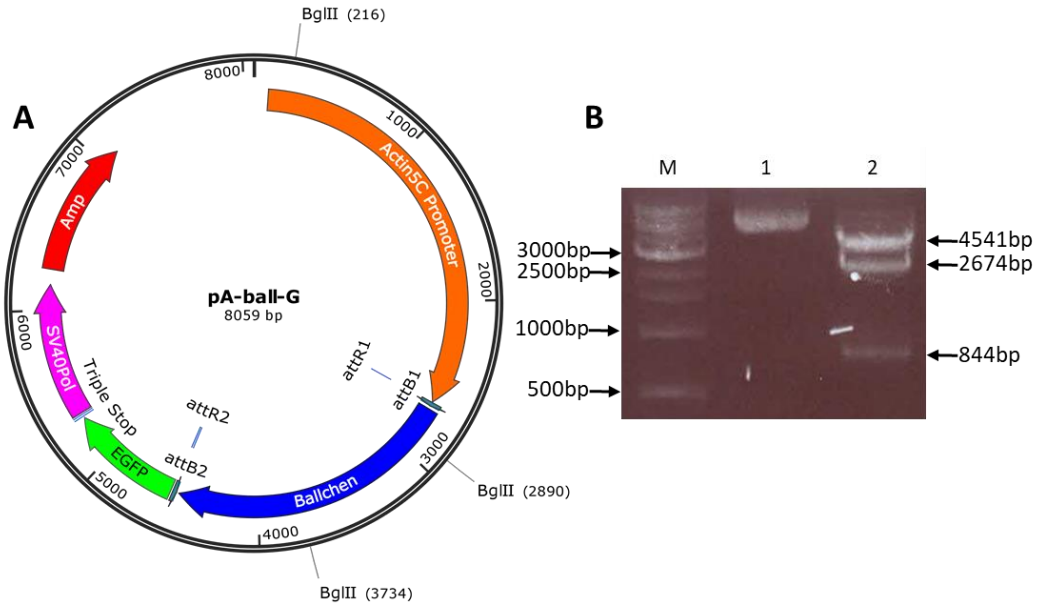

Figure 4.16: Molecular cloning of *ballchen* tagged with EGFP. (A) Schematic of GFP tagged *ball* construct where *ball-EGFP* expression is under constitutively active *Actin5C* promoter. (B) Successful cloning was confirmed by restriction digestion with BglII that led to the release of three bands. The expected sizes of the bands were 4541bp, 2674bp and 844bp. The restricted plasmid (lane 2) was resolved along with unrestricted plasmid (lane 1) and 1kb DNA marker (M).

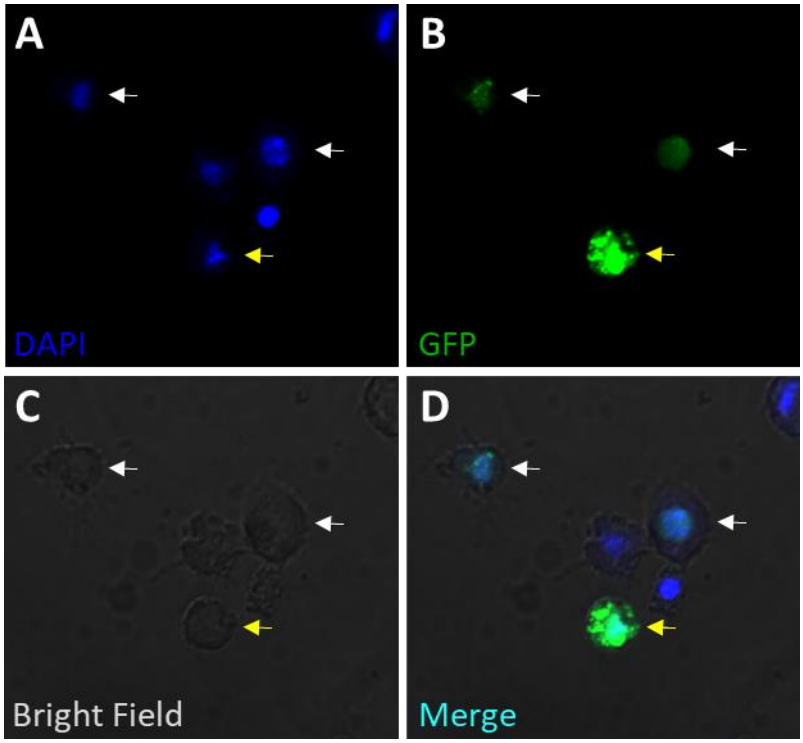

Figure 4.17: Ball subcellular localization in S2 cells. Transiently transfected S2 cells with construct expressing *GFP*-tagged *ball* under constitutively active *Actin5C* promoter showed Ball localization in either only the nucleus (white arrows) or in both cytosol and nucleus (yellow arrow). (A) DAPI staining, (B) GFP signal, (C) Bright field image and (D) represents the merge image of (A, B and C).

4.9 Ball localization on polytene chromosomes

4.9.1 Ball binds to polytene chromosomes

After confirmation of Ball localization in the nucleus in transiently transfected cells, it was analyzed if Ball binds to chromatin. Association of Ball with chromatin was investigated using polytene chromosomes which are giant chromosomes present in salivary glands of the third instar larvae. These chromosomes start as normal chromosomes in development but become enormously large due to repeated DNA replication without cell division at third instar larval stage and are ideal to visualize protein binding to chromosomes. Immunostaining of polytene chromosomes using antibody against endogenous Ball revealed that Ball indeed binds to chromatin at several loci (**Figure 4.18**).

4.9.2 Ball co-localize with Trx and Pc on polytene chromosomes

Next, it was analyzed if Ball co-localizes with Trithorax (Trx) on polytene chromosomes or both proteins bind in a mutually exclusive manner. Since both Ball and Trx antibodies were raised in rabbits, co-staining of endogenous Ball and Trx could not be performed. However, to achieve this objective, a *Drosophila* transgenic line containing *UAS-ball-EGFP*, that expressed Ball specifically in the salivary glands with the help of *Sgs-GAL4* driver line, was used (**Appendix G**). Immunostaining of polytene chromosomes from salivary glands of third instar larvae expressing *ball-EGFP* revealed a substantial overlap between Ball and Trx (**Figure 4.19**). It was also visible that both Ball and Trx have several binding sites that are mutually exclusive.

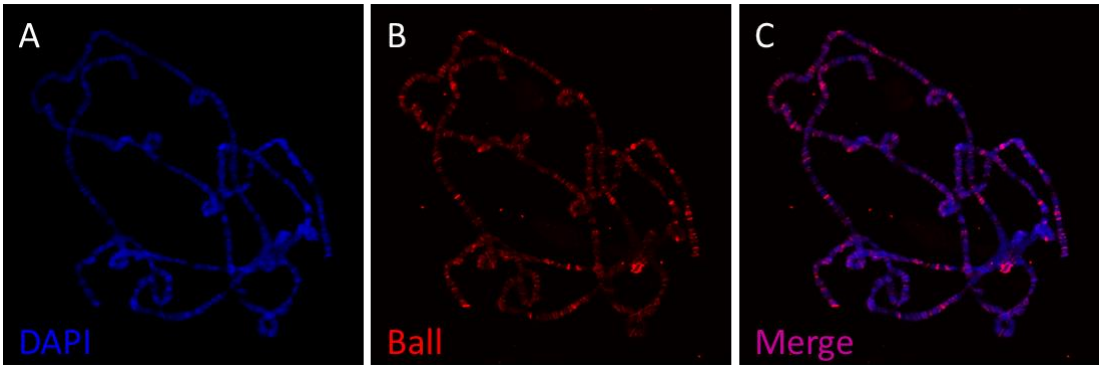

Figure 4.18: Ball localizes to polytene chromosomes. Immunostaining with anti-Ball antibody shows association of Ball at several loci on polytene chromosomes. (A) DAPI staining, (B) anti-Ball staining and (C) Merge.

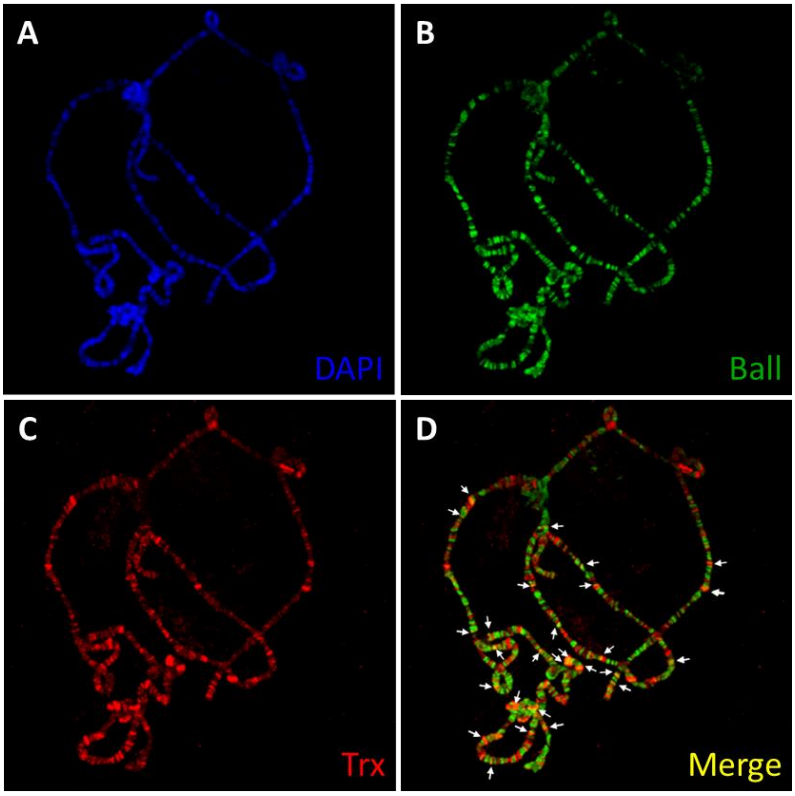

Figure 4.19: Ball partially co-localizes with Trx on polytene chromosomes. Third instar larvae expressing GFP-tagged Ball were used to stain polytene chromosomes with anti-GFP (B) and anti-Trx (C) antibodies, while (A) represents the DAPI staining. The overlap between Ball and Trx is observable at several loci (D, white arrows).

Next, it was investigated whether Ball also co-localizes with Polycomb protein (Pc) on the polytene chromosome. To this end, third instar larvae specifically expressing *UAS-ball-EGFP* in the salivary glands were used for co-immunostaining of polytenes with Pc and Ball-EGFP. Both Pc and Ball were found to co-occupy several loci on polytenes (**Figure 4.20**). Nevertheless, the overlap between Ball and Pc occupancy is not complete. There are other loci also noticeable, where Ball or Pc binds exclusively without the presence of the other.

Since Ball associates with both Trx and Pc, it was investigated whether association of Ball with Pc/Trx has anything to do with active or silent states of gene expression. On polytene chromosomes, nine loci are extensively transcribed if a heat shock at 37^{o}C is given to third instar larvae (**Methodology Section 3.4.3**). Due to high levels of transcription, such polytenes show puff like appearance (Ashburner and Bonner, 1979). Trx protein is also known to localize along with high levels of RNA Pol-II at these loci after heat shock (Shopland and Lis, 1996; Smith et al., 2004). To know if Ball co-localizes with Trx at these heat shock puffs, immunostaining of polytene

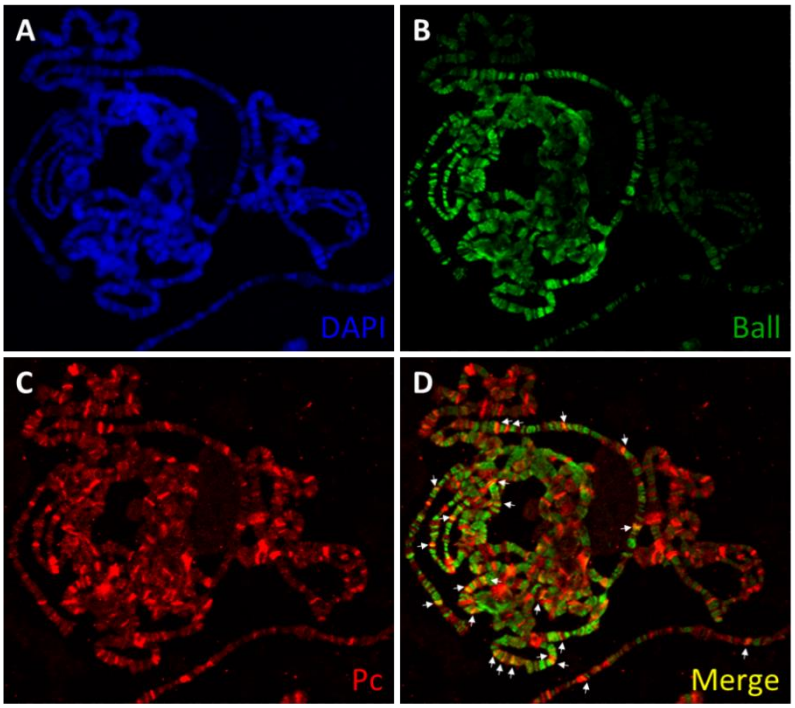

Figure 4.20: Ball partially co-localizes with Pc on polytene chromosomes. Polytene chromosomes from third instar larvae expressing EGFP-tagged Ball stained with DAPI (A), anti-GFP (B) and anti-Pc (C) antibodies. Co-localization of Ball and Pc is seen at several loci (D, white arrows).

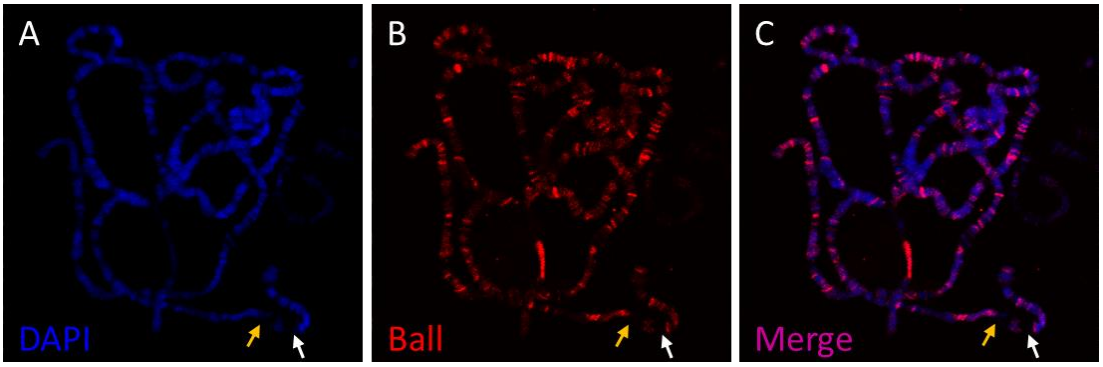

Figure 4.21: Ball does not occupy heat shock puffs. Immunostaining of polytene chromosomes isolated from heat-shocked third instar larvae revealed Ball does not localize at heat shock puffs (A-C). DAPI staining is shown in (A) and anti-Ball staining is shown in (B). (C) depicts the merge of Ball and DAPI staining. White arrow corresponds to 95D and yellow arrow represents 93D cytological positions on 3R chromosome.

chromosomes with the anti-Ball antibody was performed before and after heat shock. No enrichment of Ball at heat shock puffs was observed at position 95D and 93D which are known sites of heat shock puffs on chromosome 3R (**Figure 4.21**).

4.10 Ball binds to trxG target loci on chromatin

To validate association of Ball on polytene chromosomes, binding of Ball on specific trxG target loci was analyzed by performing chromatin immunoprecipitation (ChIP).

To this end, first *ball* CDS was cloned in-frame with 8x His and 3x FLAG epitope tags under *metallothionein* (MT) promoter to generate *pMT-ball-HF* construct (**Figure 4.22A, B**). This copper inducible promoter was preferred to specifically induce *ball* expression and avoid constitutive over-expression in cells. Gateway recombination based cloning method (**Methodology Section 3.6**) was used to generate this construct, which was confirmed by double digestion with BglII and HindIII restriction enzymes (**Figure 4.22C**). Double digestion released three expected fragments of 6701bp, 844bp and 464bp sizes, confirming the successful cloning (for details see **Appendix B**). *Drosophila* S2 cell line was used to generate stable cells that contain *pMT-ball-HF* construct. Western blot analysis of these stable cells using an anti-FLAG antibody detected expected Ball protein, which confirmed the stable integration of the construct into the genome (**Figure 4.22**).

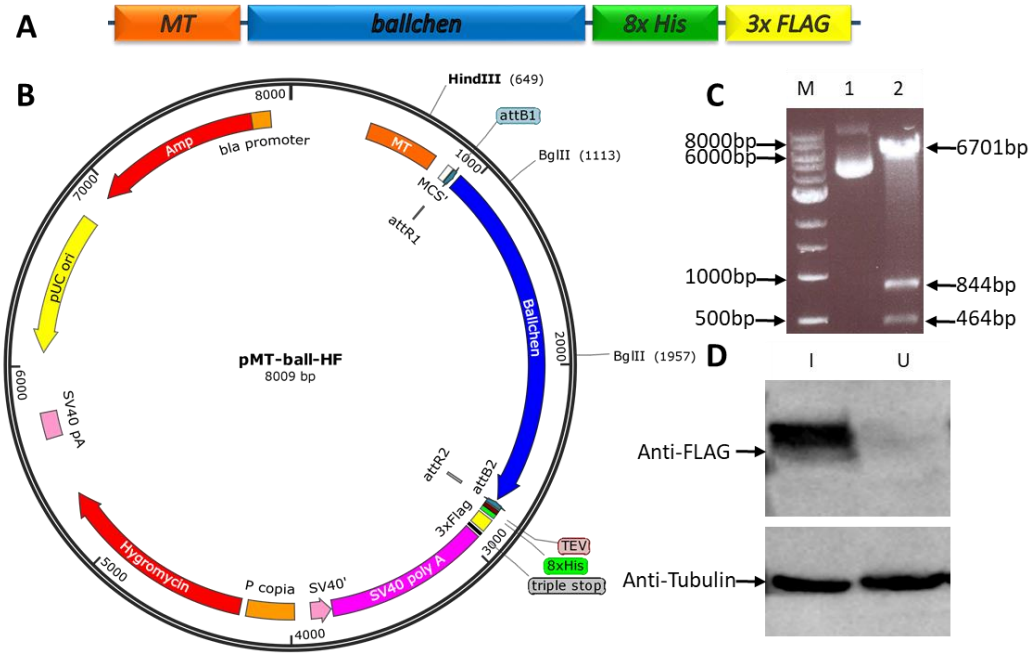

Figure 4.22: Molecular cloning of *ball* in-frame with *His* and *FLAG* tags (*pMT-ball-HF*) under copper inducible promoter. (A) Schematic of *His* and *FLAG*-tagged *ball* where *ball* coding sequence is fused in-frame with coding sequences of 8X *His* and 3X *FLAG* epitopes, under copper inducible promoter (*MT*). (B) Graphic representation of the vector backbone containing *pMT-ball-HF* along with Hygromycin resistance gene. (C) The construct was confirmed through restriction digestion with BglII and HindIII that resulted in the release of expected bands of 6701bp, 844bp and 464bp sizes. The restricted plasmid (lane 2) was resolved along with unrestricted plasmid (lane 1) and 1kb DNA marker (M). (D) Western blot analysis of stable cell line expressing FLAG-tagged *ball* coding sequence under copper inducible promoter. The cell lysate prepared from copper-induced cells (I) after 72 hours from induction was resolved along the cell lysate prepared from un-induced cells (U), while Tubulin was used as the loading control.

Next, ChIP was performed with anti-FLAG antibody using formaldehyde fixed chromatin from these cells. The purified DNA after ChIP was used to perform qRT-PCR using primers specific for trxG binding sites in homeotic and non-homeotic genes. Ball was found at the transcription start sites (TSS) of *pipsqueak* (*psq*), *pannier* (*pnr*), *pointed* (*pnt*) and *disconnected* (*disco*) which are known binding sites of PcG/trxG (**Figure 4.23**). Moreover, the association of Ball was also observed at *iab-7PRE*, *bxd* and *Dfd* regulatory regions of homeotic genes (**Figure 4.23**), known to be bound by both Pc and Trx proteins. Ball was present at chromatin regardless of active or silent states of genes in this analysis. Importantly, Ball was also seen at an intergenic region, which is normally not occupied by PcG/trxG (see IR in **Figure 4.23**). In contrast, ChIP from empty vector control cells resulted in negligible enrichment at all the regions analyzed. Binding of Ball on trxG target loci was further confirmed by performing ChIP with the antibody against endogenous Ball (**Figure 4.23**). Similar results but with even stronger enrichment of Ball were obtained for all homeotic as well as non-homeotic targets that were analyzed.

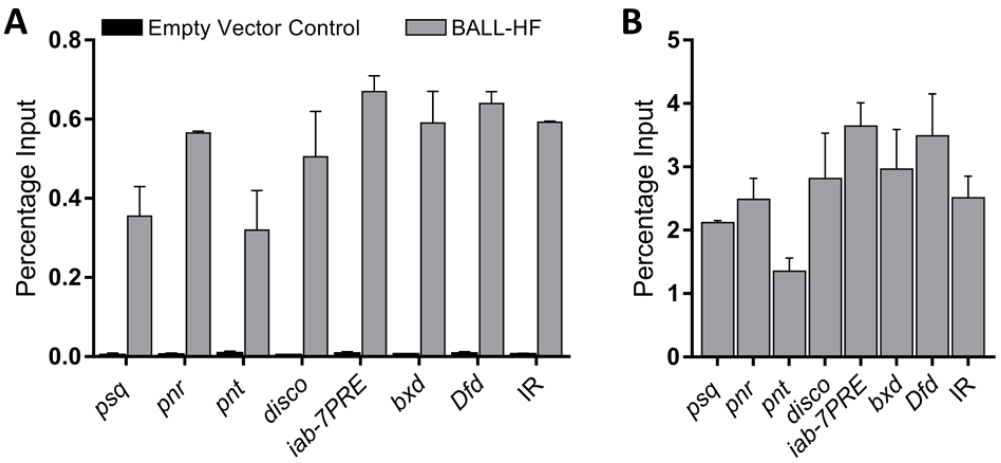

Figure 4.23: Ball is strongly enriched at trxG target loci. (A) ChIP using an anti-FLAG antibody in *Drosophila* S2 cells expressing FLAG-tagged Ball (Ball-HF). Strong Ball enrichment was observed at all loci analyzed in ChIP-qPCR. ChIP-qPCR from an empty-vector control cell line using the anti-FLAG antibody was used as the negative control. (B) ChIP from S2 cells using an antibody against endogenous Ball also displayed a similar pattern but stronger enrichment.

4.11 Ball inhibits PRC1 mediated H2AK118 ubiquitination

Ball is a nucleosomal histone kinase that has a known primary function of phosphorylating histone H2A at threonine position 119 (H2AT119) (Aihara et al., 2004). This threonine residue is adjacent to a lysine residue at 118 position of histone H2A (H2AK118). The ubiquitination of H2AK118 (H2AK118ub1) is a histone mark that is widely associated with PcG mediated silencing of genes. H2AK118ub1 is a covalent modification catalyzed by an E3 ubiquitin ligase dRING which is the catalytic subunit of the PRC1 complex (Wang et al., 2004). It was postulated that by phosphorylating H2AK119 (H2AK119p) Ball may inhibit H2AK118 ubiquitination and counteract PRC1 mediated repression. To analyze whether the H2AK119ub1 mark is indeed enriched at PcG/trxG targets in absence of Ball, ChIP from Ball depleted cells was performed using an antibody against H2AK118ub1. ChIP from cells treated with dsRNA against *LacZ* was used as control. Both active (*pnt*, *pnr*) and silent targets (*bxd*, *Dfd*) of PcG/trxG showed an increase in H2AK118ub1 enrichment in cells where the *ball* is knocked down as compared to control cells (**Figure 4.24**). Depletion of Ball leading to increased enrichment of H2AK118ub1 also corroborates with decreased *pnt* and *pnr* expression where *ball* is knocked down as described previously in (**Figure 4.7**).

These results suggest that Ball association with trxG at chromatin counteracts PcG mediated repression by inhibiting H2AK118ub1, a histone modification catalyzed by PRC1. This synergistic behavior of Ball/trxG nexus explains genetic and molecular interactions observed between Ball and trxG. It also adds a new dimension to the anti-silencing act of trxG that counteracts PcG to maintain epigenetic cell memory during development.

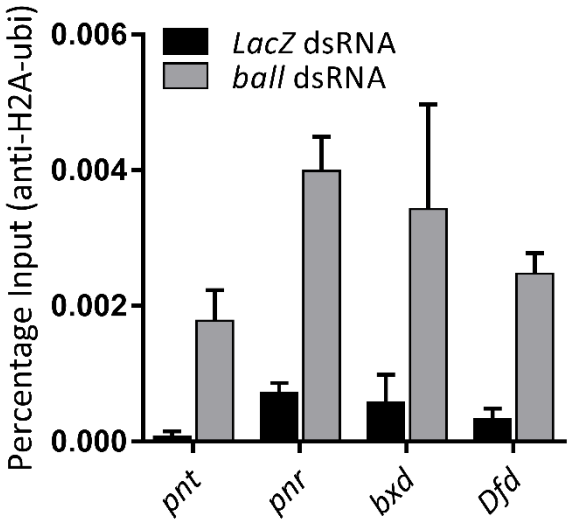

Figure 4.24: H2AK118 ubiquitination increased in Ball depleted cells. ChIP using an anti-H2A-ubi antibody in *Drosophila* D.Mel-2 cells exhibit strong enrichment of H2AK118ub1 in Ball depleted cells as compared to cells treated with dsRNA against *LacZ*.

5 DISCUSSION

Despite an in-depth understanding of cell signaling pathways at the cellular and molecular level and their pivotal role in the life of a cell, still little is known about how cell signaling components effect cell fate maintenance. The first kinase was discovered more than half a century ago (Krebs and Fischer, 1955) along with phosphorylation reaction itself (Burnett and Kennedy, 1954), but the role of kinases and associated proteins in regulating heritable gene expression has remained elusive. The work presented in this dissertation aimed to decipher a molecular link between epigenetic cell memory and cell signaling pathways. A kinome-wide RNAi screen was performed to identify cell signaling kinases and associated proteins that are involved in trxG mediated gene activation. A stringent criterion, based on the Z-scores of *trx* and *ash1* knockdowns, was applied to shortlist twenty-eight genes from the kinome-wide screen. Among these candidate genes presence of *fs(1)h*, the only canonical trxG member with predicted kinase activity, indicates the potential of the screen to discover new trxG regulating kinases. The human homolog of Fs(1)h, BRD4 not only binds to acetylated histones in enhancer and promoter region and interacts with other trxG proteins but also phosphorylate RNA Pol-II at serine 2 of tandem repeats of heptapeptide sequence present in its C-terminal domain (CTD) to promote transcription elongation (Devaiah et al., 2012). Unlike its mammalian homolog, *Drosophila* Fs(1)h is not yet known to phosphorylate serine 2 of the tandem repeat sequence in CTD of RNA Pol-II. Interestingly, CDK12 which is the top candidate in our primary screen is already known to phosphorylate RNA Pol-II CTD tandem repeat at serine 2 position in *Drosophila* (Bartkowiak et al., 2010). Many other trxG interacting proteins and histone modifiers were also present in the shortlisted candidate genes. For example, Skittles, a nuclear phosphatidylinositol 4-phosphate 5-kinase, is one of the candidates known to biochemically interact with Ash2 which is a trxG member (Cheng and Shearn, 2004). Additionally, Skittles mutants not only have downregulation of Ubx expression in the third leg and haltere imaginal discs but also enhance the homeotic transformation phenotype of both Ash1 and Ash2 (Cheng and Shearn, 2004). The catalytic product of Skittles, phosphoinositol-4,5-bisphosphate (PIP4,5), is also known to stabilize mammalian

BAF (Brahma associated factors) complex on chromatin (Zhao et al., 1998). PIP4,5 is also known to bind with the linker histone H1 and induce its phosphorylation that results in H1 dissociation from chromatin on silent loci. Consequently, H1 dissociation from chromatin positively impacts RNA Pol-II activity (Yu et al., 1998), thus directly linking a signaling secondary messenger with chromatin regulation.

CDK2, another candidate from the screen, is known to phosphorylate H1 and disrupt its direct interaction with HP1 (Hale et al., 2006). Phosphorylation of histone H1 mimics the loss of H1 and induces transcription. Unlike other histones, the number of phosphorylated sites rather than specific phosphorylated residues is important in case of H1 (Dou and Gorovsky, 2000). The Cyclin E which binds CDK2, also physically interacts with Brahma and Moira, the core proteins of BAP/PBAP chromatin remodeling complexes (Brumby et al., 2002). CDK2, Cyclin E together with Decapo, the cyclin-dependent kinase inhibitor (CKI) of CDK2-Cyclin E complex, all behaved as trxG genes in our screen. It is known that CDK2-Cyclin E complex phosphorylates Brahma homolog BRG1 in mammals to inhibit its function (Shanahan et al., 1999). Similarly, in *Drosophila*, constitutive phospho-mimic mutant of Brahma that has all potential CDK target serine/threonine residues mutated into aspartic acid acts as a loss of function mutation while phospho-blocking mutant of Brahma that has all potential CDK target sites mutated into alanine acts as a gain of function mutation (Roesley et al., 2018). Our screen reveals additional possibilities of how Cyclin E, CDK2, Decapo and BAP complex may function in cellular context with respect to each other.

Protein kinases also affect epigenetic cell memory by causing post-translational modifications like phosphorylation of PcG proteins. For instance, EZH2 in mammals is phosphorylated by multiple kinases at multiple sites which result in either enhanced (Yap et al., 2011) or decreased enzymatic activity (Wei et al., 2011), translocation from nucleus to cytoplasm (Anwar et al., 2018), its proteasomal degradation (Sahasrabuddhe et al., 2015) and change its interaction with histones (Cha et al., 2005). Two of our candidate kinases CDK1 and CDK2 are shown to phosphorylate EZH2 *in vitro* to either decrease its methyltransferase activity or to maintain H3K27me3 mark in daughter cells during mitosis (Chen et al., 2010; Kaneko et al.,

2010; Zeng et al., 2011). A model predicting phosphorylation-dependent binding of EZH2 with its recruiters and subsequent H3K27me3 on newly synthesized H3 is also proposed (Chen et al., 2010). In agreement with our study, phosphorylation of EZH2 by CDK1 and CDK2 not only stresses upon roles of kinases in epigenetic cell memory but also links cell cycle with it. Recently, CDK1 is reported to phosphorylate a plethora of histone writers and erasers. MLL2, LSD1, G9a, SUV39H2, SETD2, DOT1L, p300, KDM2A, HDAC6 are some important histone modifiers included in the list of CDK1 substrates among many others. Interestingly, both DNA methyltransferase DNMT1 and erasers Tet1 and Tet2 are also phosphorylated by CDK1 (Michowski et al., 2020).

Cell cycle regulation is a unifying feature of many of our candidates as suggested by their STRING and Reactome analysis. Such involvement of cell cycle regulators in cell memory maintenance corroborates with the gradual decrease of PRC2 mediated H3K27me3 levels with each round of cell division if the PRC2 recruiting reporter sequence is removed. H3K27me3 levels remained high otherwise, in replication-stalled cells (Laprell et al., 2017). Recently, differential recruitment of PRC2 subunits to cell lineage-specific promoters is also reported across cell cycle in mouse embryonic stem cells (mESCs) (Asenjo et al., 2020). My data not only strengthen the notion that PcG mediated gene repression is regulated in cell cycle dependent manner but also pinpointed the regulators of cell cycle that may play a crucial role in modulating the cellular function of PcG/trxG system.

Apart from cell cycle regulation, another unifying feature of some of the kinases in our list of candidates is their known role in the phosphorylation of histones. For example, AurB, BubR1 and Ball are three histone kinases from the list of candidates that phosphorylate different residues in either N-terminal tail of histone H3 or C-terminal tail of histone H2A. H2A C-terminal tail is the substrate for PRC1 (Wang et al., 2004) and is the only C-terminal unstructured tail among core histones (Luger et al., 1997). On the other hand, the N-terminal tail of histone H3 is substrate for major complexes of PcG as well as trxG, including PRC2, COMPASS, COMPASS-like, TAC1 and Ash1 (Zhao and Shilatifard, 2019). AurB is known to phosphorylate histone H3 at serine 10 (Crosio et al., 2002) and serine 28 (Goto et al., 2002), both of which are

adjacent to two covalently modified residues in H3 tail, which are known hallmarks of silent chromatin (H3K9me3 and H3K27me3). The post-translational modifications (PTMs) on neighboring residues of histones are proposed to antagonize each other and termed as binary switches in gene regulation (Fischle et al., 2003). Binary switches of H3K9me3/H3S10p and H3K27me3/H3K28p have already been proposed and demonstrated to regulate gene expression in heterochromatin and epigenetic cell memory, respectively (Fischle et al., 2005; Gehani et al., 2010; Hirota et al., 2005).

Apart from phosphorylating histone H3, AurB also phosphorylates H2A C- terminal tail at threonine 119, adjacent to PRC1 mediated H2AK118ub1 mark, but in centromeric regions (Brittle et al., 2007). The mammalian homolog of BubR1 (Bub1) too phosphorylates the same residue in the centromeric and pericentromeric regions (Kawashima et al., 2010; Maeda et al., 2018). Ball, on the other hand, phosphorylates H2AT119 in chromosomal arms (Aihara et al., 2004; Brittle et al., 2007). Similar to Ball, its mammalian homolog VRK1 is also known to phosphorylate H2A in promoter regions (Aihara et al., 2016). Ball involvement in the cell cycle, signal transduction and its molecular function of modifying histone residues in chromosomal arms at promoters and not in the centromeric regions made it the best candidate for dissecting its role in PcG/trxG mediated epigenetic memory.

Although Ball is a histone kinase that modifies histone H2A, it was never associated with the maintenance of gene activation by trxG or epigenetic cell memory prior to our kinome-wide RNAi screen. Our results revealed a strong genetic interaction of *ball* mutant with *Pc* and *trx* mutations. The mutation in *ball* not only strongly suppresses the extra sex comb phenotype of *Pc* mutants but also enhances the *trithorax* mutant phenotype. This is further substantiated by the fact that target genes of PcG/trxG are also downregulated in *ball* homozygous mutant embryos. At the molecular level, this downregulation of trxG target genes in *ball* depleted fly embryos or cells can be explained by the presence of Ball at trxG targets on chromatin. Ball also promotes H3K4me3 and H3K27ac, the histone marks associated with gene activation. H3K4me3 is considered a global gene activation mark rather than a mark specific to trxG mediated gene activation (Ardehali et al., 2011; Hallson

et al., 2012). Nonetheless, Ball facilitates H3K4me3 that emphasizes on its role in gene activation. On the other hand, H3K27 is associated with trxG mediated anti-silencing activity that further substantiates Ball involvement in maintenance of transcriptional cell memory (Tie et al., 2009, 2014). Such interplay of Ball with histone acetylation also corroborates with previously reported data that suggests depletion of VRK1, the mammalian homolog of Ball, leads to loss of H3K14 acetylation and H4 acetylation (Salzano et al., 2015). Together, all these results suggest that Ball exhibits trxG like behavior and its presence at trxG targets is required for the maintenance of gene activation. Moreover, these results also support a notion that H2AT119p catalyzed by Ball antagonizes PRC1 mediated H2AK118ub1 and consequent repression. Recently, it is reported that its PRC1 mediated H2A ubiquitination that plays the central role in PcG mediated gene repression (Blackledge et al., 2020; Tamburri et al., 2020), which further signifies Ball role in cell memory and brings Ball to the center of PcG/trxG mediated gene regulation system. These findings suggest that the inhibitory effect of Ball on H2AK118ub1 positively contributes to the anti-silencing activity of trxG.

Based on the results presented in this dissertation, I propose a histone H2AK118ub1/H2AT119p binary switch in *Drosophila* that regulates the maintenance of epigenetic cell memory governed by PcG/trxG system (**Figure 5.1**). Histone H2A C-terminal tail, along with the N-terminal tail of histone H3, leaves nucleosome near the entry-exit site of DNA (Luger et al., 1997). The amino acids stretch of histone H2A from position 115 to 122 passes between the strands of DNA wrapped around nucleosome (Luger et al., 1997) and the addition of phosphate on this stretch of amino acid can biochemically favor unwrapping of DNA. The presence of H2A C-terminal tail in close vicinity to histone H3 N-terminal tail at the entry-exit site of DNA in the nucleosome also hints towards a cross-talk between histone modifications by PcG, trxG and Ball. The DNA interactions with H3 N-terminal tail and H2A C-terminal tails act as two main barriers in the initiation of DNA unwrapping (Ettig et al., 2011) and phosphorylation can weaken such interactions by increasing net negative charge of histone tails. Interestingly, VRK1, the mammalian homolog of Ball is known to phosphorylate histone H3 at both Threonine 3 and serine 10

positions in addition to histone H2A at threonine 120 position (Kang et al., 2007). The role of histone phosphorylations by VRK1 in epigenetic cell memory and in particular PcG/trxG system warrants further investigation.

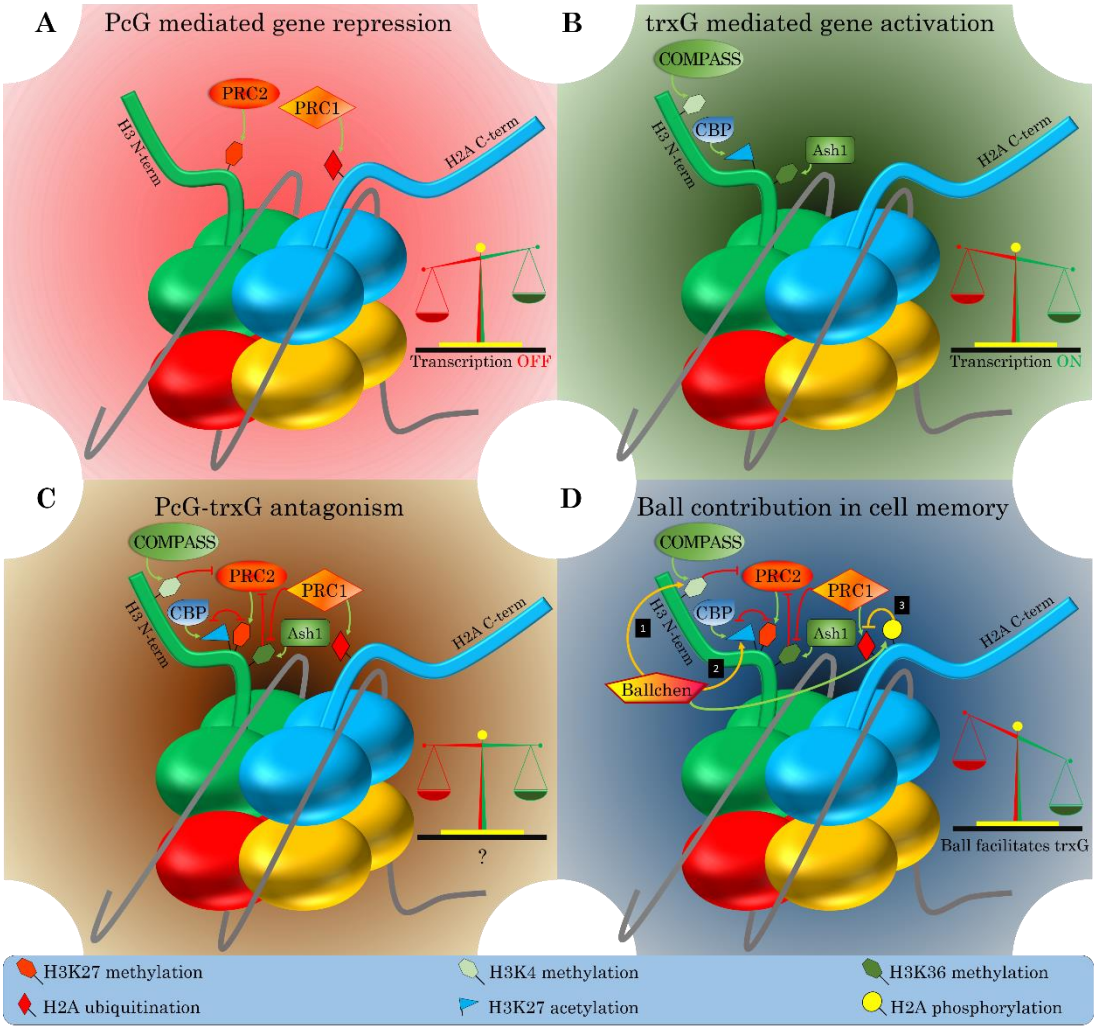

Figure 5.1: Proposed model of Ball, facilitating trxG mediated gene activation and antagonizing PcG mediated transcription repression. (A) PcG proteins mediate gene repression through PRC2 associated H3K27 methylation and PRC1 mediated H2A ubiquitination. (B) Gene activation by trxG through COMPASS complexes mediated H3K4 methylation, CBP mediated H3K27 acetylation and Ash1 mediated H3K36 methylation. (C) PcG and trxG antagonize each other and inhibit histone marks deposited by the other group. H3K4 and H3K36 methylation marks inhibit PRC2 catalytic activity and variant of PRC1 demethylate H3K36 via KDM2. Methylation and acetylation marks upon the same H3K27 residue also inhibit each other. (D) Ball contributes to the transcriptional control by facilitating H3K4 methylation (1) and H3K27 acetylation (2), that feed into the complex interplay of trxG and PcG. Ball mediated H2A phosphorylation is also proposed to inhibit H2A ubiquitination (3), the hallmark of PcG mediated gene repression.

5.1 Limitations of the study

- The cutoff for identifying cell signaling components involved in cell memory maintenance was based on the Z-scores of known trxG members that makes the identification criterion very stringent. But at the same time, such stringent criterion could also fail to identify some of the cell memory regulators and generate false negatives
- Although the primary screen was validated by the secondary screen but in both screens the efficiency of knockdown was not determined because it is difficult to know how efficient is a specific dsRNA in large scale screens. This may result in failure of identifying all the regulators of trxG mediated gene activation.
- The kinome-wide RNAi screen revealed twenty-eight genes that may contribute to maintenance of cell fate but only effect of Ball was further characterized for its role in trxG mediated cell memory. Many other genes in the list of candidates, as discussed in the previous section, may have the potential of revealing novel cell signaling-cell memory nodes if further characterized.
- Ball facilitates trxG in mediating gene activation but such interplay can be context dependent as not all the targets of trxG were downregulated in Ball depleted embryos. Partial overlap of Ball and Trx as well as Ball and Pc occupancy on polytenes also suggest Ball interaction with PcG/trxG system is context dependent. In the absence of a genome-wide binding profile of Ball, it is difficult to conclude exact relationship between Ball and trxG.
- Ball promotes H3K4me3 and H3K27ac histone marks but the mechanism by which it acts is missing and demands further investigation.
- Although a model is proposed that suggests histone H2AK118ub1/H2AT119p binary switch in this dissertation and H2AK118ub1 enrichment is observed upon *ball* knockdown but direct evidence for the existence of such switch is still lacking.

5.2 Future directions

Molecular understanding of the role of Ball in the maintenance of gene activation by trxG provides novel insights in role of a signaling kinase in epigenetics. Importantly, many of the candidate genes revealed in kinome-wide screen in this dissertation have the potential to provide major insights into how transcriptional cellular memory is maintained. In the case of Ball, a detailed mechanistic understating of its molecular function linked to epigenetic memory also needs further scrutiny as VRK1, the mammalian homolog of Ball, has substrates other than histones that interact with PcG/trxG system. For example, a substrate of VRK1 is Sox2 which is known to counteract epigenetic repression by PRC2 in neural progenitor cells (Amador-Arjona et al., 2015). VRK1 and Sox2 are known to cooperate during cell cycle progression and form a stable protein complex (Moura et al., 2016). Similarly, CREB is phosphorylated by VRK1 at serine 133 (Kang et al., 2008) and this specific phosphorylation is known to enhance CREB interaction with CBP that in turn increase CREB target genes transcription (Dyson and Wright, 2005; Radhakrishnan et al., 1998). Such interaction of VRK1 and CREB also corroborates with the depletion of H3K27ac in *ball* mitotic clones discovered in this study. Furthermore, the transcription factor from the JNK signaling pathway, c-Jun, is also phosphorylated by VRK1 at the same residues that are targeted by JNK (Sevilla et al., 2004a). Intriguingly, it has been shown in *Drosophila*, that the over-expression of JNK signaling pathway downregulates Polycomb mRNA expression (Lee et al., 2005; Roumengous et al., 2017). The aforementioned signaling nodes need further investigation to elucidate Ball/VRK1 role in the maintenance of epigenetic cell memory.

As stated earlier, the mammalian homolog of Ball, VRK1 also phosphorylates histone H3T3 that is a transient mitotic histone modification, involved in chromatin condensation during the cell cycle (Kang et al., 2007). Working as a binary switch, H3T3 phosphorylation is known to inhibit binding of TFIID (Varier et al., 2010), CHD1 (Flanagan et al., 2005), MLL5 (Ali et al., 2013), DIDO and ING1 (Gatchalian et al., 2016) to H3K4me3. Similar to H3S28p which protects H3K27me3 from demethylases (Kruidenier et al., 2012; Sengoku and Yokoyama, 2011), it also protects H3K4me3

from KDM4A demethylase (Su et al., 2016). As the phosphorylation of H3T3 strongly correlates with mitosis (Polioudaki et al., 2004), detailed investigation should be carried out to understand the role of Ball/VRK1 in protection of H3K4me3 by VRK1 mediated H3T3 phosphorylation during mitosis.

The role of phosphatases with respect to Ball/VRK1 mediated maintenance of epigenetic states of gene expression also needs to be probed as Trx is known to physically interact with type 1 serine/threonine protein phosphatase (PP1). Homeotic transformations by Trx mutations are suppressed by PP1 mutation (Rudenko et al., 2003) that was initially characterized as a suppressor of variegation (Baksa et al., 1993). The mammalian homolog of PP1 is shown to dephosphorylate the histone phosphorylations caused by VRK1 (Hsu et al., 2000; Qian et al., 2011).

Some of the contrasting downstream functions of histone modifications carried out by candidate kinases from the kinome-wide RNAi screen are yet another theme that must be explored further to understand signaling to the chromatin and epigenetics of gene regulation. For example, on one hand, Ball is known to be involved in condensation of chromatin during cell cycle yet it is also counteracting the PcG gene repressors. Similarly, both histone marks of H2AT120p and H3T3p, catalyzed by VRK1, are known to activate another candidate kinase AurB (Kelly et al., 2010; Wang et al., 2010a; Yamagishi et al., 2010) but contrarily, AurB and VRK1 are known to cross-inhibit each other (Moura et al., 2018). AurB is known to phosphorylate H3S28 that too on one hand protects the H3K27me3 mark from demethylases (Kruidenier et al., 2012; Sengoku and Yokoyama, 2011) and on the other hand, it is also known to inhibit PcG proteins recruitment to H3K27me3 (Lau and Cheung, 2011). Interestingly, the PcG mediated H2AK118ub1 mark is known to inhibit AurB mediated histone phosphorylation (Joo et al., 2007). How such contrasting functions are carried out by the same proteins and histone modifications and how such interactions are interrelated are some questions that demand more detailed analysis at molecular and biochemical level. It is proposed to decipher this intricate relation by investigating role of these proteins linked to their involvement in cell cycle as well as novel roles they may play beyond cell cycle.

As discussed earlier, histone H2A C-terminal tail leaves nucleosome from the entry-exit site of DNA, it also acts as a recognition module for linker histone H1 (Vogler et al., 2010). How phosphorylation of H2A C-terminus affects the H1 recognition of nucleosome is of special significance in the context of epigenetic cell memory due to the following three arguments. Firstly, the histone H1 is enriched in silent chromatin where methylated H3K9 and H3K27 are enriched as well as depleted at actively transcribing promoters where enhanced levels of H3K4 methylation are found (Cao et al., 2013). Secondly, H1 is known to directly interact with human PRC2 subunits (Martin et al., 2006). Thirdly, H1 is also enriched at hypoacetylated regions in chromatin (Reczek et al., 1982; Schrother et al., 1981) and it negatively regulates histone acetyltransferases (Herrera et al., 2000). Additionally, H1 also interacts with HP1 (Hale et al., 2006) and a histone deacetylase, SirT1 (Vaquero et al., 2004), that further explains its association with hypoacetylated silent chromatin.

The entry-exit site of DNA in nucleosome serves as a dynamic platform that integrates many biological processes including epigenetic cell memory. Approximately 30% of transcription factors binding sites in yeast are located in the entry-exit site stressing upon its importance in regulating gene transcription (North et al., 2012). Any PTM, including H2A C-terminus phosphorylation and ubiquitination near this region, would not only affect unwrapping of DNA but also can affect transcription factors binding. To enhance our understanding of genome accessibility via histone phosphorylations and their writers, investigation of structural information is the key. Techniques like single-molecule fluorescence resonance energy transfer (FRET), small-angle X-ray scattering (SAXS) and atomic force microscopy (AFM) should be utilized to observe the effects of histone phosphorylation on nucleosome dynamics. Hybrid structural biology approaches that integrate crystallography, cryo-electron microscopy (cryo-EM), nuclear magnetic resonance (NMR) and molecular dynamics simulations can help us answer the difficult question of how the histone kinases and subsequent phosphorylations effect the multiprotein PcG/trxG complexes binding to chromatin or its modification.

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7 APPENDICES

7.1 Appendix A: *Drosophila* as model organism

Drosophila melanogaster is a powerful model organism that was first brought to light by Thomas Hunt Morgan in 1910 when he confirmed the chromosome theory of inheritance using *Drosophila* (Morgan, 1910). Today, all modern *Drosophila* researchers can trace back their scientific lineage to Morgan. The common name of *Drosophila* is fruit fly that belongs to the *Diptera* order and *Drosophilidae* family (Powell, 1997). Easy maintenance inside the lab, short life cycle, fast reproduction and amplification, compactness of genome and availability of many intelligent manipulative genetic tools are some of the reasons that made this organism a very effective and attractive model to work with. Here, a comprehensive outline of the advantages of *Drosophila* will be given that are most relevant to this dissertation.

7.1.1 Short life cycle

The short life cycle of *Drosophila* was the feature that attracted early 20th century scientists to use it for their advantage. It requires only 9 to 10 days for the development of adult fly from a fertilized egg at 25^{o}C that make fast production of multiple generations within a short time span possible (Ashburner, 1989). Fertilized eggs require around 24 hours to develop into first instar larva which is the first developmental stage after embryogenesis. first instar larval stage lasts for another 24 hours before transformation into second instar larvae. Similar to the transition from first instar to second instar, transformation into third instar larvae from second instar stage requires yet another day. The last developmental stage before puparation is third instar larvae stage that requires 2 more days of growth before the transition. Pupae stage lasts for about 5 more days before metamorphosis from pupae to adult fly. The advantage of a short life cycle further increases by the fact that it can also be slowed down to some extent according to researchers' needs by controlling the temperature of the cultured flies. At 18^{o}C, the life cycle takes about 19 days to complete, making *Drosophila* rapid enough for fast-paced research and convenient enough to work with, at the same time (Ashburner, 1989).

7.1.2 Ease of designing crossing schemes

An ideal genetic model organism should be able to generate inbred stocks that can carry mutations, insertions and other manipulative sequences. Such stocks should also remain stable across multiple generations. Being a model genetic organism, *Drosophila* can have stable stocks of different genotypes as well as it can undergo multigenerational crossings between different stable stocks easily. Setting up a cross is as simple as collecting unmated females (remain virgin for 8 hours after hatching) and mixing them with males of the desired genotype. A strong advantage of using *Drosophila* is the existence of balancer chromosomes that make crossing schemes easier to design and carry out. Balancer chromosomes have three key features that are: 1. Balancer chromosomes carry visible dominant marker mutations, 2. Balancer chromosomes carry multiple inversions for preventing recombinant offspring survival, 3. Balancer chromosomes also carry a recessive lethal mutation for preventing its own selection in an inbred population. Balancer chromosomes make the generation of inbred stable stocks, selection of desired genotypes and designing of meticulous crossing schemes much easier for the researcher (Greenspan, 1997).

7.1.3 Compact genome

Drosophila melanogaster is being used as a model organism for more than a century now. That means extensive work for genome curation and development of ingenious genetic tools has already been done. The genome size of *Drosophila* is about 180 megabases (Bosco et al., 2007), out of which 120 megabases constitute euchromatin (Adams et al., 2000; Celniker et al., 2002). *D. melanogaster* genome is made up of only four chromosome pairs out of which chromosome number 4 is a very small and gene-poor autosome that is often neglected. Chromosome numbers 2 and 3 (autosomes) and the X chromosome (sex chromosome) carry most of the genes as the Y chromosome is also comparatively gene-poor and small. Because of the small genome size and extensive usage in labs, *Drosophila melanogaster* became the second multicellular organism, after *Caenorhabditis elegans* (1998b), whose whole genome sequencing is published (Adams et al., 2000; Celniker et al., 2002; Hoskins et al., 2007).

7.1.4 UAS-GAL4 expression system

In *Drosophila*, expression of any gene of interest can be achieved in any particular tissue or at any developmental stage using the ingenious expression system of UAS-GAL4. GAL4 is a transcriptional factor of yeast that can bind to a specific DNA sequence called UAS (upstream activating sequence). For utilizing this bipartite expression system in *Drosophila*, GAL4 expression is controlled in spatial/ temporal manner within a fly that has a construct, integrated into its genome, containing UAS enhancer connected with the coding sequence of a specific gene (UAS-specific gene). In this fashion, the specific gene under UAS control can be expressed in the tissue of choice or developmental stage of choice. To control the expression of GAL4, fly stocks, called driver lines, are generated that have GAL4 under various endogenous transcriptional enhancers. When any of such driver line is crossed with a responder line (UAS- specific gene), the progeny has the expression of the specific gene under the endogenous transcriptional enhancer used for expressing GAL4 in the driver line. In short, the UAS-GAL4 system is facilitating the researchers of the fly community to do rescue experiments, targeted knockdown of genes of interest in specific tissues and many other manipulative experiments (Brand and Perrimon, 1993; Duffy, 2002).

7.1.5 Mitotic clones

To study genes that are essential for early development in *Drosophila*, another innovative genetic manipulation is the production of mosaic organisms that have homozygous mutant cells in patches within a heterozygous background. Mitotic recombination is employed to generate such mosaic fly stocks by utilizing the Flp-FRT recombination system. Flippase (Flp) is a recombinase enzyme from yeast that is used to act on specific DNA sequences called FRT sites. The first transgenic fly with a Flippase enzyme was generated in 1989 in which it is under the control of *Heat shock protein 70* (*Hsp70*) promoter. Mitotic recombination can be induced by simply raising the temperature at any specific developmental stage of the organism (Golic and Lindquist, 1989). Flippase can also be controlled by a tissue-specific promoter to induce mitotic recombination in a specific tissue of interest. To generate homozygous mutant clones for a gene of interest, the mutation is introduced on a chromosome arm carrying FRT site. The other homologous chromosome also carries

the FRT site at the same locus but with a wild-type copy of the gene of interest. Besides, the *wild-type* gene carrying chromosome is marked with a constitutive marker (often ubiquitously expressing GFP construct). Once the mitotic recombination event occurs in a cell, the subsequent cell division produces daughter cells with two different genotypes which are, the cell that is heterozygous for the mutation and expressing GFP and the cell that is a homozygous mutant and not expressing GFP. Upon further cell divisions, both the cells will generate patches of cells of their respective genotype, thus creating viable somatic clones of an otherwise lethal mutation in a heterozygous animal (Beira and Paro, 2016).

7.1.6 Cell lines

Drosophila is a model organism for genetic studies but it also has certain advantages in cell culture over other systems. Out of around a hundred different cell lines of *Drosophila* origin, S2 and Kc are the most commonly used cell lines (Echalier and Ohanessian, 1970; Schneider, 1972). One of the most common uses of the S2 cell line is expression and purification of recombinant proteins where the prokaryotic expression system fails to provide a soluble or active form of recombinant protein (Davis et al., 1993; Ikonomou et al., 2003). Another advantage of S2 cells is that transfection efficiency is higher than most other cell lines. Unlike mammalian cell lines, the *Drosophila* cell line also has the ability to absorb dsRNA of longer length (500-1000bp) and then chopping long dsRNA into short pieces, making it more suitable for knocking down specific genes and analyzing effects of such knockdown. To knock down a specific gene effectively, *Drosophila* cells are simply required to be bathed with dsRNA against that gene (Echeverri and Perrimon, 2006). Our study particularly took advantage of this trait of *Drosophila* cell culture to perform the kinome-wide RNAi screen.

7.2 Appendix B: General procedures and chemicals

Plasmid transfection for kinome-wide screen

	Vol. µl/well (µl)	5600
pPRE-F.Luc (100 ng/µl)	0.02	112
Actin-R.Luc(100ng/µl)	0.03	168
EC buffer	4.81	26936
add Enhancer in ratio: 1 µg DNA in 8 µl		
Enhancer	0.04	224
mix, incubate 2-5 min and add		
Effectene	0.1	560
incubate 5 - 10 min		
Express Five Medium	5	28000
Mix well but gently		
add 10 µl per well		

Plasmid transfection for secondary screen

	Vol. µl/well (µl)	81x
DNA (pPRE-FLUC: pActin RLUC=5:1, total: 50 ng/µl)	2	162
EC buffer	28	2268
add Enhancer in ratio: 1 µg DNA in 8 µl		
Enhancer	0.8	64.8
mix, incubate 5 min		
Effectene+EC buffer	2.5+17.5	202.5+1417.5
Incubate 3-4 min		
Mix well but gently		
Mix both with each other, incubate for 15 minutes		
add 50 µl per well		

10X PBS (pH 7.4) and 1X PBS

Components	Amount (g/L)
Potassium Phosphate monobasic (KH_2PO_4)	2.4
Potassium Chloride (KCl)	2
Sodium Chloride (NaCl)	80
Sodium Phosphate dibasic (Na_2HPO_4)	7.2g
Distilled Water	Volume adjusted to 1L
To make 1X PBS dilute 1:10	
To make 1X PBST, Tween 20 was added in 0.1% final concentration	

PEM Buffer

Components	Amount (/L)
PIPES	0.1M
$MgCl_2$	1mM
EGTA	1mM
Adjust pH to 6.9 with KOH	

Buffers for immunostainings of imaginal discs

Blocking Buffer	0.3% Triton X-100 + 5%BSA in 1X PBS
Buffer 1	0.1% Triton X-100 + 1%BSA in 1X PBS

Buffers for immunostainings of polytenes

1X PBST	1X PBS with 1%Triton X-100
Blocking Buffer	5% non-fat milk in 1X PBS
Antibody buffer	1%BSA, 2% goat serum in 1X PBS
Wash Buffer 300	300mM NaCl, 0.2% NP-40, 0.2% Tween20 in 1X PBS
Wash Buffer 400	400mM NaCl, 0.2% NP-40, 0.2% Tween20 in 1X PBS

End point PCR reaction for Actin

Components	Amount
PCR water	14.75µl
10X PCR buffer	2µl
10mM dNTPs	1µl
Forward primer (10µM)	0.5µl
Reverse primer (10µM)	0.5µl
Polymerase	0.25µl
Template	1µl (~75ng)

Primer melting temperature for Actin is 58^{o}C and for ball amplification 60^{o}C. For PCR amplification of templates for dsRNAs, 60 ^{o}C melting temperature was used.

Restriction Digestion with BglII

Ingredient	Volume µl
BglII (thermos scientific)	0.5 (5 units)
10X Buffer O	2
PCR water	14.5
DNA	3 (1-5µg)
Total	20

Restriction Digestion with BglII and HindIII

Ingredient	Volume µl
BglII (thermos scientific)	0.5 (5 units)
HindIII (thermos scientific)	05 (5 units)
10X Buffer R	2
PCR water	14
DNA	3 (1-5µg)
Total	20

Hoyer's medium

15g gum arabic was added to 25ml ddH20 in a beaker and was placed on a heat plate with stirring overnight at 60°C. Next day, chloral hydrate (100g) was added in small batches with stirring, followed by the addition of 10g glycerol. The mixture was centrifuged at max speed for half an hour and supernatant was carefully transferred to a 50ml falcon tube and sealed tightly.

7.3 Appendix C: Solutions for ChIP

Solutions for chromatin immunoprecipitation

Solution A **Solution B** **10X Fixation Solution** **Sonication Buffer** **2.5M Glycine**	For sonication of chromatin prepared fresh
5M NaCl	For reverse cross-linking
0.5M EDTA (pH8) **1M Tris-Cl (pH 6.5)** **Phenol Chloroform Isoamyl Alcohol** **1X TE** **Glycogen (concentrated)** **3M Sodium Acetate (pH 5.2)** **Absolute Ethanol**	For extraction of chromatin
1X RIPA for ChIP **2X RIPA for ChIP** **1X Lithium Chloride Buffer** **Elution Buffer**	For pulldown in ChIP
10% SDS **1M Tris (pH8.0)** **Triton X-100** **0.5M EGTA** **10% Sodium deoxycholate**	For making solutions above

Details of Solutions for ChIP

Solution A	**200ml**
10mM Tris (pH8)	2ml of 1M
0.25% Triton X-100	500µl of 100%
10mM EDTA	4ml of 0.5M
0.5mM EGTA	200 µl of 0.5M
Water	193.3ml
Solution B	**200ml**
10mM Tris (pH8)	2ml of 1M
200mM NaCl	8ml of 5M
1mM EDTA	0.4ml of 0.5M
0.5mM EGTA	200 µl of 0.5M
Water	189.4ml
10X Fixative Solution	**200ml**
1M NaCl	40ml of 5M
50mM Tris (pH 8)	10ml of 1M
10mM EDTA	4ml of 0.5M
5ml EGTA	2ml of 0.5M
Water	144ml

Note: 1X fresh each time. Final concentration of 37% Formaldehyde should be EXACTLY 1%.

Sonication Buffer	**200ml**
10mM Tris pH8	2ml of 1M
1mM EDTA	0.4ml of 0.5M
0.5mM EGTA	0.2ml of 0.5M
Water	197.4ml
2.5M Glycine	50ml
Mr: 75.07	
Mass needed for 50ml	9.38g

Note: Filter sterilize all the solutions. Store at 4ºC.

1X TE	**40ml**
10mM Tris (pH 8)	400µl of 1M
1mM EDTA	80µl of 0.5M
Water	Up to 40ml
3M Sodium Acetate pH 5.2	50ml
Mr: 136.08	
Mass needed for 50ml	20.4g – Adjust pH to 5.2
2X RIPA for ChIP	**200ml**
20mM Tris	4ml of 1M
2mM EDTA	0.8ml of 0.5M
280mM NaCl	11.2ml of 5M
2% Triton X-100	4ml
0.2% SDS	4ml of 10%
0.2% Sodium deoxycholate	4ml of 10%
Water	172ml
1X RIPA for ChIP	
Dilute from 2X RIPA as needed. 1:1.	
Lithium Chloride Buffer	**100ml**
10mM Tris pH 8	1ml of 1M
250mM LiCl	1.06g of solid (Mr = 42.39g)
1mM EDTA	0.2ml of 0.5M
0.5% NP-40	714µl of 70%
0.5% Sodium deoxycholate	5ml of 10%
Water	93.1ml
Elution Buffer (Fresh each time)	**10ml**
0.1M Sodium Bicarbonate ($NaHCO_3$)	0.084g
1% SDS	1ml of 10%
Water	Up to 10ml

Dissolve sodium bicarbonate in ~8ml of water. Then add the SDS. Make up the volume to 10ml. If you add SDS before sodium bicarbonate has dissolved there is a lot of foaming which would lead to an error in making up the final volume.

For dilution of fixative solution

	For 5ml (scaled down)	For 40ml (scaled up)
10X Fixative Solution	3.55ml	28.4ml
37% Formaldehyde	1.45ml	11.6ml

Protease inhibiters to be added freshly in solution A, B, sonication buffer, RIPA buffer, TE buffer and lithium chloride buffer

Stock concentration	Final Concentration
PMSF (100mM)	1mM
Pepstatin (1µg /µl)	2µg /ml
Aprotinin (1µg /µl)	2µg /ml
Leupeptin (1µg /µl)	2µg /ml

7.4 Appendix D: Solutions for western blotting

Western Blotting Solutions

12% SDS-PAGE		
Resolving gel	**Components**	**Total volume 20ml**
	Milli Q Water	6.6ml
	30% Acrylamide	8ml
	1.5M Tris (pH 8.8)	5ml
	10% SDS	0.2ml
	10% APS	0.2ml
	TEMED	0.008ml
Stacking gel	**Components**	**Total volume 5ml**
	Milli Q Water	3.4ml
	30% Acrylamide	0.83ml
	1 M Tris (pH 6.8)	0.63ml
	10% SDS	0.05ml
	10% APS	0.05ml
	TEMED	0.005ml
Tris-Glycine running buffer (5X)	**Components**	**Total volume 1L**
	Tris-base	15.1g
	Glycine	72g
	SDS (10%)	5g
Transfer buffer (10X)	**Components**	**Total volume 1L**
	Glycine	144g
	Tris-base	30.25g
1X transfer buffer	**Components**	**Total volume 1L**
	10X transfer buffer	100ml
	Methanol	200ml
	Distilled water	700ml

7.5 Appendix E: Sequencing data analysis

Sequencing analysis of *ball* cloning

Base Number	Base changed	Varient Type	Amino Acid changed	Amino Acid Number
51	T to C	missense	S to P	18
77	G to A	synonymous	N/A	N/A
188	G to C	synonymous	N/A	N/A
212	C to G	missense	N to K	71
275	C to T	synonymous	N/A	N/A
749	C to A	synonymous	N/A	N/A
820	C to T	missense	P to L	274
959	C to T	synonymous	N/A	N/A
992	G to T	synonymous	N/A	N/A
998	G to A	synonymous	N/A	N/A
1018	A to G	missense	N to S	340
1114	A to G	missense	K to R	372
1301	C to T	synonymous	N/A	N/A
1373	G to A	synonymous	N/A	N/A
1381	C to A	missense	A to D	461
1419	C to A	synonymous	N/A	N/A
1421	T to G	synonymous	N/A	N/A
1430	G to T	synonymous	N/A	N/A

7.6 Appendix F: Primers list for knocking down genes

Name gene	Name Primer	Amplicon ID	Amplicon Size	Off-Targets	Hits	Primers Sequence
Pk17E (S6KL)	S6KL_F19349	DRSC19349	263	0	0 of 93	TAATACGACTCACTATAG**AACATCAAACGCCACCTACT**
	S6KL_R19349	DRSC19349				TAATACGACTCACTATAG**CTCAGGTTATAGTCCTTGTAGA**
Pk17E (S6KL)	S6KL_F23147	DRSC23147	475	0	2 of 60	TAATACGACTCACTATAG**CAACATCAAACGCCACCTAC**
	S6KL_R23147	DRSC23147				TAATACGACTCACTATAG**TTGTAAGGCTCCTCCAGCTC**
polo	polo_F37001	DRSC37001	329	0	0 of 28	TAATACGACTCACTATAG**AATTCTGCAAGGCAATCTGG**
	polo_R37001	DRSC37001				TAATACGACTCACTATAG**TGTTCACATTGTTGGCACCT**
polo	polo_F38944	DRSC38944	258	0	0 of 22	TAATACGACTCACTATAG**GCAAAACCGAGATTTGATCG**
	polo_R38944	DRSC38944				TAATACGACTCACTATAG**GTAGGTTTTCCGCTGGTTGA**
cdc2(CDK1)	cdc2_F03504	DRSC03504	577	0	24 of 94	TAATACGACTCACTATAG**AGTCGGGTAGCGAAGTAAC**
	cdc2_R03504	DRSC03504				TAATACGACTCACTATAG**GTCTGTTTGGAGGATGTTTTG**
cdc2(CDK1)	cdc2_F40860	DRSC40860	334	0	1 of 7	TAATACGACTCACTATAG**GAATAGCGGCTTTCTCGTTG**
	cdc2_R40860	DRSC40860				TAATACGACTCACTATAG**GCTGCCAGTTGATAAGCACA**
Drak (CG32666)	Drak_F28871	DRSC28871	308	0	2 of 44	TAATACGACTCACTATAG**CCACAACAACAGCAACAACC**
	Drak_R28871	DRSC28871				TAATACGACTCACTATAG**GTGGCGACTGGCTTCTTTAC**
Drak (CG32666)	Drak_F36990	DRSC36990	413	0	0 of 11	TAATACGACTCACTATAG**TCGGTGTGTGTTCAATCGTT**
	Drak_R36990	DRSC36990				TAATACGACTCACTATAG**CACACAAACGTTTCGCCTAA**
ial (aurora B)	aurB_F23324	DRSC23324	384	0	0 of 60	TAATACGACTCACTATAG**CGATGGATCACGTTGTTCAG**
	aurB_R23324	DRSC23324				TAATACGACTCACTATAG**TCCCCGGGACTTTGAGAT**
ial (aurora B)	aurB_F03548	DRSC03548	506	4	7 of 94	TAATACGACTCACTATAG**CAGCCGACGACGAACTC**
	aurB_R03548	DRSC03548				TAATACGACTCACTATAG**GCCATGAAGGTGATGTTCAAA**
cdc2c(CDK2)	cdc2c_F16921	DRSC16921	185	0	9 of 94	TAATACGACTCACTATAG**TATGCGCCGCTCCTTG**
	cdc2c_R16921	DRSC16921				TAATACGACTCACTATAG**CGTGCGCCTCGTGTTC**

cdc2c(CDK2)	cdc2c_F36625	DRSC36625	353	0	1 of 12	TAATACGACTCACTATAG**GTCATTTCCGGCAACAATCT**
	cdc2c_R36625	DRSC36625				TAATACGACTCACTATAG**CCTTGCTAGGCCAAAGTCAG**
Sik2 (CG4290)	Sik2_F18522	DRSC18522	492	0	1 of 93	TAATACGACTCACTATAG**AGTCTTCGGTGCTCTGTTT**
	Sik2_R18522	DRSC18522				TAATACGACTCACTATAG**AAAGACTTAGCCGCCATCA**
Sik2 (CG4290)	Sik2_F36760	DRSC36760	447	0	0 of 11	TAATACGACTCACTATAG**GCTCCTTGAGTTTGAGCAGG**
	Sik2_R36760	DRSC36760				TAATACGACTCACTATAG**GCTCTCCGTGTGAGATTTCC**
CDK4	CDK4_F27263	DRSC27263	214	0	1 of 44	TAATACGACTCACTATAG**GTATGCCAGTTTCTGGAGCG**
	CDK4_R27263	DRSC27263				TAATACGACTCACTATAG**CCGGTCAGTAGTTCCCTTGA**
CDK4	CDK4_F07358	DRSC07358	321	4	5 of 94	TAATACGACTCACTATAG**CTTGTCAAGGGAACTACTGAC**
	CDK4_R07358	DRSC07358				TAATACGACTCACTATAG**ATGCGGTCCAGCTGATTC**
wit	wit_F26091	DRSC26091	510	0	1 of 49	TAATACGACTCACTATAG**ACACTCCATCAAAGGCAAGG**
	wit_R26091	DRSC26091				TAATACGACTCACTATAG**AGCCAGTCGGAATGTACCAG**
wit	wit_F37034	DRSC37034	398	0	0 of 16	TAATACGACTCACTATAG**AGGTTTATCGAGGCGAGGAT**
	wit_R37034	DRSC37034				TAATACGACTCACTATAG**CTCTGACAATGCGAACTCCA**
Hipk	Hipk_F23155	DRSC23155	404	0	0 of 60	TAATACGACTCACTATAG**ACCAGATCTACAACGGACGC**
	Hipk_R23155	DRSC23155				TAATACGACTCACTATAG**GAGGTGGTGCTTGGGATAGA**
Hipk	Hipk_F36886	DRSC36886	339	0	0 of 16	TAATACGACTCACTATAG**CGTGGACTCAACGTATCCCT**
	Hipk_R36886	DRSC36886				TAATACGACTCACTATAG**CACACCTCCATCATCTGCAC**
Ball	DRSC26607 F	DRSC26607	341	0	1 of 46	TAATACGACTCACTATAGGGAGA**ATCCTGGTCCGCTTTCTTTT**
	DRSC26607 R	DRSC26607				TAATACGACTCACTATAGGGAGA**ATAGTTCACCACCCAGCCAG**
BubR1	DRSC33237 F	DRSC33237	528	0	-	TAATACGACTCACTATAGGGAGA**ATTGTTTGGAAAGACACCGC**
	DRSC33237 R	DRSC33237				TAATACGACTCACTATAGGGAGA**TCTGACATGGAATCGTTGGA**
LacZ F						TAATACGACTCACTATAGGGAGA**GGAAGATCAGGATATGTGG**
LacZ R						TAATACGACTCACTATAGGGAGA**CTTCATCAGCAGGATATCC**

7.7 Appendix G: Fly strains

Table 7.1: The mutant fly strains used for genetic analysis of *ball* mutant with their Flybase ID, mutation type and the mutagen used for generating mutation.

Alleles	Flybase ID	Mutation type	Mutagen
Pc^{1}	FBal0013551	amorphic allele of *Polycomb* gene	X-Ray
Pc^{XL5}	FBal0013561	amino acid number 69 and 70 deleted in Chromo domain of *Polycomb*	X-Ray
ball^{2}	FBal0299570	deletion of translation start codon and part of kinase domain of *ballchen*	Imprecise excision of P-element insertion
w^{1118}	FBal0018186	5' half of the *white* gene is deleted	spontaneous
trx^{E2}	FBal0017174	amorphic allele of *trx* gene	gamma-ray
trx^{1}	FBal0017165	9kb insert in first intron of *trx* gene	spontaneous

Table 7.2: The fly strains used for generating somatic clones of *ball^{2}* along with their genotype and description (Herzig et al., 2014; Yakulov et al., 2014).

Name	Genotype	Description
HsFlp	*HsFlp; +; +*	*Flipase* under heatshock protein 70 promoter
Tb/Sb	*w*; +; TM6 Tb/ TM3 Sb*	third chromosome balancer fly line
FRT82B, Ubi-GFP	*+; +; P{neoFRT}82B, P{Ubi-GFP}*	FRT recombination site at cytological position 82B of third chromosome with GFP marker.
Ball^{2}/Sb	*+; +; P{neoFRT}82B, ball^{2}/TM3 Sb*	Mutated *ballchen* copy with FRT sites at cytological position 82B, balanced with *TM3*.Stubble

Table 7.3: The fly genotypes generated through genetic crosses along with their descriptions.

Genotype	Descriptions
HsFlp; +; TM6 Tb/ TM3 Sb	Flipase under *Hsp70* promoter present on X chromosome. third chromosome is also balanced.
HsFlp; +; P{neoFRT}82B, P{Ubi-GFP}	Flipase under *Hsp70* promoter on X chromosome and FRT recombination site at cytological position 82B of third chromosome with GFP marker present in same fly.
+; +; P{neoFRT}82B, ball^{2}/TM6 Tb	Mutated *ballchen* copy with FRT sites at cytological position 82B balanced with *TM6 Tubby.*

Table 7.4: The GAL4 line, RNAi lines and balancer strains used in this dissertation along with their descriptions, Flybase ID, and genotype.

Strain	Flybase ID	Descriptions	Genotype
UAS-Dicer; en-Gal4, UAS-GFP	FBst0025752	Gal4 expressed under engrailed promoter with GFP marker. Dicer expression for efficient knockdown	*P{UAS-Dcr-2.D}1, w^{1118}; P{en2.4-GAL4}e16E, P{UAS-2xEGFP}AH2*
UAS Dicer; enGAL4 myr RFP/CyO		Gal4 expressed under engrailed promoter with RFP marker. Dicer expression for efficient knockdown	*UAS Dicer; enGAL4 myr RFP/CyO*
Sgs-GAL4	FBst0006870	Expresses GAL4 in salivary glands.	*w^{1118}; P{Sgs3-GAL4.PD}TP1*
Tb/Sb	FBal0018186	Balancer fly strain for third chromosome	*w^{1118}; TM3 Sb/TM6 Tb*
P{ActGFP}JMR2	FBst0004534	Balancer fly strain for third chromosome with GFP signal	*w^{1118}; Sb^1/TM3, P{ActGFP}JMR2, Ser^1*
Ball RNAi1	FBst0055330	For knockdown of *ball in vivo*	*y^1 v^1; P{TRiP.HMC04017}attP4*
Ball RNAi2	FBst0035571	For knockdown of *ball in vivo*	*y^1 sc^* v^1; P{TRiP.GL00068}attP2}*

7.8 Appendix H: Genes analyzed in kinome-wide RNAi screen

Flybase IDs of the 433 genes knocked down in kinome-wide screen				
FBgn0000017	FBgn0038504	FBgn0031696	FBgn0019949	FBgn0011285
FBgn0000063	FBgn0038542	FBgn0031730	FBgn0019957	FBgn0011300
FBgn0000116	FBgn0038588	FBgn0031784	FBgn0019990	FBgn0011598
FBgn0000147	FBgn0038603	FBgn0031855	FBgn0020386	FBgn0011737
FBgn0000150	FBgn0038630	FBgn0031860	FBgn0020389	FBgn0011739
FBgn0000179	FBgn0038736	FBgn0031995	FBgn0020391	FBgn0011754
FBgn0000229	FBgn0038816	FBgn0032006	FBgn0020412	FBgn0011817
FBgn0000242	FBgn0038902	FBgn0032083	FBgn0020440	FBgn0011829
FBgn0000258	FBgn0038952	FBgn0032147	FBgn0020621	FBgn0013435
FBgn0000259	FBgn0039015	FBgn0032180	FBgn0020622	FBgn0013759
FBgn0000273	FBgn0039083	FBgn0032187	FBgn0020767	FBgn0013762
FBgn0000274	FBgn0039179	FBgn0032363	FBgn0020930	FBgn0013955
FBgn0000395	FBgn0039306	FBgn0032424	FBgn0021796	FBgn0013984
FBgn0000442	FBgn0039403	FBgn0032456	FBgn0022029	FBgn0013987
FBgn0000479	FBgn0039623	FBgn0032633	FBgn0022131	FBgn0013988
FBgn0000489	FBgn0039673	FBgn0032650	FBgn0022338	FBgn0013997
FBgn0000536	FBgn0039692	FBgn0032677	FBgn0022382	FBgn0014001
FBgn0000721	FBgn0039790	FBgn0032752	FBgn0022708	FBgn0014006
FBgn0000723	FBgn0039796	FBgn0032811	FBgn0022709	FBgn0014073
FBgn0000826	FBgn0039809	FBgn0032955	FBgn0022800	FBgn0014870
FBgn0001079	FBgn0039908	FBgn0032999	FBgn0022936	FBgn0014930
FBgn0001624	FBgn0039924	FBgn0033183	FBgn0023081	FBgn0015024
FBgn0002413	FBgn0040056	FBgn0033244	FBgn0023083	FBgn0015129
FBgn0002466	FBgn0040298	FBgn0033383	FBgn0023169	FBgn0015772
FBgn0002567	FBgn0040335	FBgn0033609	FBgn0024150	FBgn0243505
FBgn0002938	FBgn0040505	FBgn0033673	FBgn0024179	FBgn0250785
FBgn0003079	FBgn0040506	FBgn0033674	FBgn0024222	FBgn0250823
FBgn0003091	FBgn0040507	FBgn0033773	FBgn0024227	FBgn0250851
FBgn0003093	FBgn0040510	FBgn0033791	FBgn0024245	FBgn0250906
FBgn0003124	FBgn0041092	FBgn0033915	FBgn0024290	FBgn0259168
FBgn0003169	FBgn0041096	FBgn0033984	FBgn0024326	FBgn0259217
FBgn0003177	FBgn0041097	FBgn0034020	FBgn0024329	FBgn0259220
FBgn0003178	FBgn0041203	FBgn0034137	FBgn0024846	FBgn0259240
FBgn0003256	FBgn0042094	FBgn0034264	FBgn0025382	FBgn0259243
FBgn0003317	FBgn0044826	FBgn0034299	FBgn0025458	FBgn0259678
FBgn0003366	FBgn0045035	FBgn0034376	FBgn0025592	FBgn0259680
FBgn0003371	FBgn0045980	FBgn0034421	FBgn0025625	FBgn0259712
FBgn0003499	FBgn0046322	FBgn0034453	FBgn0025640	FBgn0259822
FBgn0003501	FBgn0046332	FBgn0034568	FBgn0025702	FBgn0259996
FBgn0003502	FBgn0046685	FBgn0034598	FBgn0025740	FBgn0015806
FBgn0003716	FBgn0046689	FBgn0034644	FBgn0025741	FBgn0016126
FBgn0003731	FBgn0046692	FBgn0034655	FBgn0025743	FBgn0016131
FBgn0003733	FBgn0047038	FBgn0034667	FBgn0025803	FBgn0016696
FBgn0003744	FBgn0050021	FBgn0034789	FBgn0025836	FBgn0016930
FBgn0003748	FBgn0050184	FBgn0034950	FBgn0025936	FBgn0016984

Continued from previous page				
FBgn0003984	FBgn0050295	FBgn0035001	FBgn0026063	FBgn0017550
FBgn0004009	FBgn0051017	FBgn0035008	FBgn0026064	FBgn0017558
FBgn0004106	FBgn0051097	FBgn0035039	FBgn0026136	FBgn0037356
FBgn0004107	FBgn0051140	FBgn0035089	FBgn0026181	FBgn0037381
FBgn0004227	FBgn0051183	FBgn0035142	FBgn0026193	FBgn0037469
FBgn0004367	FBgn0051643	FBgn0035266	FBgn0026323	FBgn0037491
FBgn0004373	FBgn0051999	FBgn0035392	FBgn0026370	FBgn0037632
FBgn0004461	FBgn0052199	FBgn0035397	FBgn0026371	FBgn0037663
FBgn0004462	FBgn0052301	FBgn0035410	FBgn0026602	FBgn0037679
FBgn0004598	FBgn0052373	FBgn0035484	FBgn0027101	FBgn0037804
FBgn0004603	FBgn0052428	FBgn0035590	FBgn0027491	FBgn0037995
FBgn0004624	FBgn0052484	FBgn0035798	FBgn0027497	FBgn0038120
FBgn0004656	FBgn0052666	FBgn0035938	FBgn0027504	FBgn0038167
FBgn0004784	FBgn0052687	FBgn0035950	FBgn0027507	FBgn0038206
FBgn0004833	FBgn0052703	FBgn0036030	FBgn0027569	FBgn0038258
FBgn0004834	FBgn0052743	FBgn0036099	FBgn0027587	FBgn0038279
FBgn0004839	FBgn0052758	FBgn0036142	FBgn0027889	FBgn0038463
FBgn0004864	FBgn0052944	FBgn0036187	FBgn0028360	FBgn0015380
FBgn0004876	FBgn0053196	FBgn0036337	FBgn0028410	FBgn0015399
FBgn0005355	FBgn0053519	FBgn0036368	FBgn0028427	FBgn0015400
FBgn0005592	FBgn0053531	FBgn0036494	FBgn0028484	FBgn0015402
FBgn0005640	FBgn0053554	FBgn0036511	FBgn0028546	FBgn0015617
FBgn0005666	FBgn0053969	FBgn0036544	FBgn0028683	FBgn0015618
FBgn0010197	FBgn0061359	FBgn0036663	FBgn0028741	FBgn0015763
FBgn0010269	FBgn0061360	FBgn0036723	FBgn0028833	FBgn0015765
FBgn0010303	FBgn0083959	FBgn0036742	FBgn0028888	FBgn0030573
FBgn0010314	FBgn0085347	FBgn0036896	FBgn0028978	FBgn0030683
FBgn0010316	FBgn0085373	FBgn0036904	FBgn0028997	FBgn0030685
FBgn0010355	FBgn0085385	FBgn0036930	FBgn0029114	FBgn0030697
FBgn0010379	FBgn0085386	FBgn0036942	FBgn0029662	FBgn0030864
FBgn0010382	FBgn0085388	FBgn0037022	FBgn0029736	FBgn0031030
FBgn0010389	FBgn0085390	FBgn0037093	FBgn0029834	FBgn0031265
FBgn0010407	FBgn0085413	FBgn0037098	FBgn0029891	FBgn0031267
FBgn0010441	FBgn0085421	FBgn0037115	FBgn0029970	FBgn0031279
FBgn0010473	FBgn0085436	FBgn0037146	FBgn0030018	FBgn0031299
FBgn0010504	FBgn0086657	FBgn0037218	FBgn0030051	FBgn0031441
FBgn0010715	FBgn0086736	FBgn0037315	FBgn0030087	FBgn0031451
FBgn0010909	FBgn0086899	FBgn0037325	FBgn0030219	FBgn0031462
FBgn0011205	FBgn0086909	FBgn0037327	FBgn0030300	FBgn0031518
FBgn0011276	FBgn0243486	FBgn0037339	FBgn0030384	FBgn0031643
FBgn0015295	FBgn0015300	FBgn0019686	FBgn0015277	FBgn0015279
FBgn0017581	FBgn0015801	FBgn0015278		

Flybase batch download tool can be utilized using this list to retrieve detailed updated information for all genes.

7.9 Appendices references

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