The Polycomb group protein Polycomblike in *Drosophila melanogaster* and its role in Polycomb group protein mediated gene repression

Diploma thesis

Lisa Harpprecht

May 21 2012 - November 21 2012

Supervised by Dr. Jürg Müller

and Prof. Dr. Thorsten Nürnberger

lisaharp@web.de registration number 3565005

max planck institute of biochemistry

EBERHARD KARLS UNIVERSITÄT TÜBINGEN

IFIB INTERFAKULTÄRES INSTITUT FÜR BIOCHEMIE

Aim of this work

In this project I aimed to reveal the function of the Polycomb group protein (PcG protein) Polycomblike (Pcl) in *Drosophila melanogaster* on other PcG proteins with regard to their role in gene repression during the development of the fly.

To achieve this, I knocked down the Pcl protein in *Drosophila* larvae and analyzed the effects in gene repression. I also knocked out Pcl protein levels in larval tissue of *Drosophila* larvae. Finally, I introduced point mutations into the DNA binding regions of Pcl to reduce the DNA binding activity of Pcl.

With these approaches I sought to clarify the role of this protein in Polycomb mediated gene repression, which is still elusive.

Declaration

I confirm that this diploma thesis was accomplished on my own with the mentioned facilities and sources and according to the Prüfungsordnung Biochemie Diplom (§ 23 i. d. Fassung vom 31. 07. 2003).

date Lisa Harpprecht

Acknowledgements

First, I would like to thank Dr. Jürg Müller for suggesting this very interesting topic, and Prof. Dr. Thorsten Nürnberger, who made it possible for me to pursue my work within this excellent research group.

This work would not have become what it is without the help of Maria and Reinhard, who never ceased to offer suggestions and support for my practical work. I am also grateful to Ana and Omer, who frequently advised me on fly-specific issues, to Jeongyoon, who provided the basis of the Polycomblike mutation assays in my thesis and to Katja, who integrate the Pcl constructs into the fly genome. I would also like to thank Ursel for supplying me with all the materials that I required for my work, and Sigrun, Friederike, Felice, Albena, Karin and Katharina for making my time in the group so enjoyable. I'm grateful for their help throughout this period.

Special thanks also go to Dominic Kotas for his help in editing this thesis.

Finally, I would particularly like to thank my parents for their unfailing patience, encouragement, and support at all times, and also my uncle for supporting me during my studies.

Last but not least I am very grateful to Martin, who regularly reminded me that there is a life outside of studying.

Abstract

Homeotic selector genes (Hox genes) are involved in the determination of the body segment identity during the development of *Drosophila melanogaster* and their expression needs to be strictly controlled. PcG proteins act as repressors of Hox genes. They keep Hox genes inactive in tissues, where they should not be expressed.

PcG proteins form different protein complexes like the Polycomb repressive complex 2 (PRC2), which has methyltransferase activity. Pcl is a component of PRC2. It has been shown recently that Pcl is required for efficient trimethylation of histone 3 Lysine 27 (H3-K27me3) by PRC2. However, the role of Pcl in H3-K27 trimethylation is still elusive.

To address this question, I knocked down Pcl protein levels in *Drosophila* larvae and I created cell clones lacking endogenous Pcl I showed that Pcl is necessary for the repression of Polycomb target genes, confirming a role in Polycomb mediated gene repression. Furthermore, chromatin immunoprecipitation (ChIP) assays showed that Pcl is specifically recruited to Polycomb responsive elements (PREs) of Polycomb target genes.

Notably, some DNA binding motifs have been found in Pcl which mediate binding to PREs. I examined the ability of Pcl to bind directly to DNA by introducing several point mutations in these regions to decrease DNA binding ability. The transgenes were introduced into the *Drosophila* genome to obtain animals expressing the point mutated Pcl protein in order to analyze the role of its DNA binding ability.

Zusammenfassung

Homeotische Gene (Hox-Gene) haben eine wichtige Funktion in der Bestimmung der Segmentidentitäten in der sich entwickelnden Larve und spielen somit eine bedeutsame Rolle in der Entwicklung von *Drosophila melanogaster*. Die Expression dieser Gene muss deshalb genau reguliert werden. Dies wird unter anderem bewirkt durch die reprimierende Funktion der PcG-Proteine. Sie halten Hox-Gene in Geweben, in denen keine Expression stattfinden soll, in einem inaktiven Zustand.

PcG-Proteine bilden Komplexe und jede dieser Komplexe übernimmt spezielle Aufgaben bei der Genrepression. Einer dieser Komplexe ist der Polycomb repressive complex 2 (PRC2) und seine Funktion ist die Methylierung von Histonen. PRC2 ist zum Teil assoziiert mit Pcl, ein PcG-Protein mit noch nicht vollständig geklärter Funktion. Man fand jedoch heraus, dass dieses Protein für eine effiziente Trimethylierung an Lysin 27 von Histon H3 (H3-K27) durch PRC2 nötig ist, was wiederum für die Repression der Gene erforderlich ist.

Um die Funktion dieses Proteins zu untersuchen, habe ich durch Knock-down- und Knock-out-Experimente die in den Zellen vorhandenen Mengen an dem Protein Pcl reduziert, beziehungsweise eliminiert und gezeigt, dass dieses Protein für die Repression der Polycomb-Zielgene unerlässlich ist und bestätigte somit, dass dieses Protein eine wichtige Komponente in der PcG-vermittelten Repression ist.

Außerdem zeigte ich mit Hilfe von Chromatin-Immunopräzipitation (ChIP) –Experimenten, dass Pcl an Polycomb Response Elements (PREs) bindet, den cis-regulatorischen Elementen in PcG-Zielgenen.

Um die kürzlich entdeckten DNA-Bindungseigenschaften des Pcl *in vivo* zu untersuchen, führte ich Punktmutationen in die DNA-bindende Domäne des Pcl-Proteins ein. Konstrukte, welche diese mutierten Proteine exprimieren, wurden in das *Drosophila*-Genom integriert. Diese Konstrukte ermöglichen es nun, die Bedeutung der DNA-Bindung für die Funktion des Pcl-Proteins in einem genetischen Experiment zu testen.

Table of contents

1 Introduction

1.1 Development of *Drosophila melanogaster*

Segmentation of the early embryo determines the body patterning of *Drosophila* during development. The segmentation is set up very early when the embryo still consists of a syncytium, which means that the embryo consists of one cell with the nucleus already separated. A cascade of steps then establishes the patterning of the developing fly. This cascade consists of successively expressed transcription factors that in turn regulate the expression of downstream genes, which also encode for transcription factors regulating further genes, et cetera. The cascade is started by morphogenic maternal mRNA. Expression of this maternal mRNA results in gradients of transcription factors, which in turn define the patterns of gap gene expression. Gap genes also encode for transcription factors, which again set up expression patterns of pair-rule genes. They, in turn, activate the expression of segment polarity genes. Subdivision into segments is done by pair-rule proteins, segment polarity genes are responsible for organizing anterior-posterior patterning within segments. The segment identity is determined through the regulated expression of segment specific Hox genes (Alberts et al. 2002).

A summarized model of the described hierarchic system of transcription factors in the embryonic cell is shown in Figure 1.

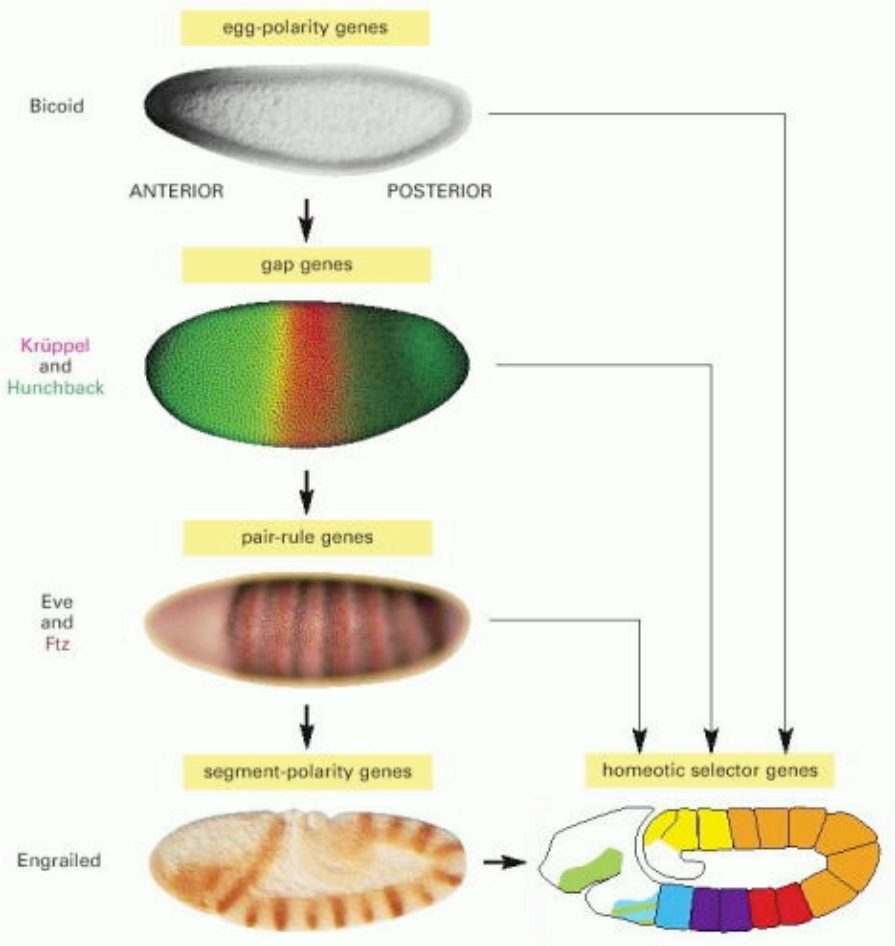

Figure 1: Hierarchic system of involved transcription factors to establish the segmentation and correct body patterning during embryogenesis of *Drosophila melanogaster*. The anterior-posterior orientation is first established by the transcription factor Bicoid, which is expressed from maternal mRNA. The resulting transcription factor gradient controls the expression of gap genes, which encodes for upstream acting transcription factors and controls the expression of pair-rule genes. These pair-rule genes encode in turn for transcription factors, which control the expression of segment-polarity genes and Hox genes. The segment-polarity genes establish the segmentation of the fly and Hox genes determine the segment identity. Pair-rule genes establish the expression pattern of Hox genes and this pattern needs to be maintained by the counteracting Trithorax group proteins and Polycomb group proteins in the late embryonic stage (not shown). Figure adopted from Alberts et al. 2002.

However, gap and pair-rule genes are only expressed in the first hours of the embryonic stage, so the established pattern needs to be maintained. This is done by two counteracting groups of protein complexes, namely the Trithorax group proteins (TxG proteins) and the Polycomb group proteins (PcG proteins). These proteins have the ability to modify chromatin to keep genes either in an activated state, or in a repressed state, respectively.

1.2 Hox genes and their role in the body patterning of *Drosophila melanogaster*

PcG proteins were first identified in fruit flies as repressors of Homeotic selector genes (Hox genes). Mutations of PcG protein encoding genes, and the consequent mis-expression of Hox genes, were shown to result in severe homeotic transformations (reviewed in Simon & Kingston 2009). Controlled expression of Hox genes is important for expression patterns of genes downstream, which are necessary for the composition of defined segments and structures during the development of *Drosophila*. McMillan provided the first indications of their existence in 1894 (Bateson 1894), but Hox genes were not discovered and classified into clusters until 1978, with Lewis's work on homeotic mutations (Lewis 1978).

Each Hox gene determines the identity of specific segments and the genes can be grouped into two complexes. The Hox genes of the Antennapedia complex specify the segments of the anterior part and the Hox genes of the Bithorax complex specify those of the posterior part of the fly. This specification leads to the development of structures in the larval stage, which are known as imaginal discs. Imaginal discs are primordia, which later on develop to form the epidermal sstructures like wings, legs or antennae in the adult. Figure 2 shows the sequence of the Hox genes and a schematic presentation of their expression domains at the extended germband stage of embyogenesis.

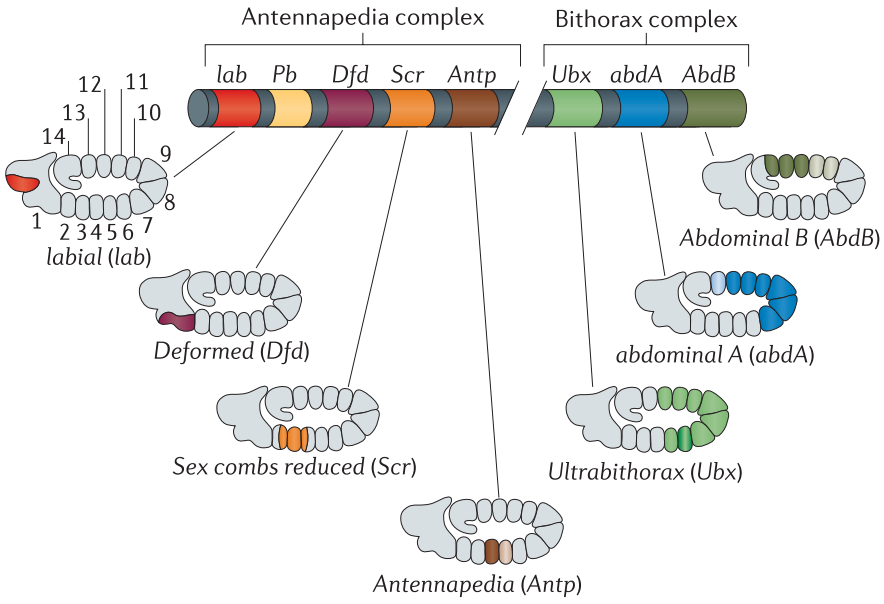

Figure 2: The identity of the segments in the fly are determined by Hox genes, whose expression pattern is established in the early embryonic stage and maintained by the counteracting Trithorax and Polycomb group proteins. The Hox genes can be grouped into the Antennapedia complex and the Bithorax complex. These complexes determine the identity of anterior segments or posterior segments of the fly. Figure adopted from Sparmann & van Lohuizen 2006.

Because of the importance of a strictly controlled Hox gene expression during fly development, PcG proteins as repressors of Hox genes have been intensively studied.

Besides their function in regulating the development in *Drosophila*, PcG proteins were shown to play an important role in the determination of flowering in plants. They are also involved in mammalian cell differentiation and therefore also may play a role in cancer (Sparmann & van Lohuizen 2006). In my thesis, however, I am not going to address these topics but concentrate on the role of PcG protein complexes and their role in fly development.

1.3 PcG protein complexes

PcG proteins act in complexes. In *Drosophila*, the PcG protein complexes Pleihomeotic repressive complex (PhoRC) an the Polycomb repressive complexes 1 and 2 (PRC1 and PRC2) and the Polycomb repressive deubiquitinase (PR-DUB) complex have been described to date and each complex has specific functions in gene repression.

E(z) is the catalytic subunit of PRC2 and trimethylates Lysine 27 of Histone H3 (H3-K27) in nucleosomes of Pcl target genes with its SET domain. This posttranslational modification is recognized by the chromodomain of Pc, a component of PRC1. PRC1, with its catalytic component Sce/Ring, ubiquitinates Lysine 119 of Histone H2A (H2A-K119), and this histone modification is involved in gene repression. PRC1 has also been shown to mediate chromatin compaction and to inhibit nucleosome remodeling (reviewed in Sparmann & van Lohuizen 2006; Simon & Kingston 2009).

PcG complexes need to be recruited to PREs. These are the cis-acting elements of PcG protein mediated gene repression and are located at Polycomb target genes. The PhoRC component Pho contains zinc finger domains and is specifically recruited to PREs. Therefore, PhoRC is thought to act as an anchor for the recruitment of the other PcG complexes to PREs (reviewed in Müller & Verrijzer 2009; Müller & Kassis 2006; Schwartz & Pirrotta 2007; Sparmann & van Lohuizen 2006).

1.4 H3-K27 trimethylation by PcG proteins

Besides H3-K27 trimethylation, E(z) can also mono- and dimethylate H3-K27. However, Papp et al. found that for repression of PcG target genes, H3-K27 trimethylation in the promoter and coding region seems to be critical. Mono- and dimethylation of H3-K27 is distributed all over the genome and is not thought to be involved in regulation of HOX gene repression by PcG proteins (Schwartz & Pirrotta 2007; Nekrasov et al. 2007). Besides this, other histone modifications are found at Polycomb target genes and are known to play a role in PcG mediated repression like trimethylation at Lysine 9 of Histone H3 or at Lysine 20 of Histone H4 (H3-K9me3 and H4-K20me3, respectively) or ubiquitination at Lysine 119 of H2A-K119 (H2A-K119ub) (Papp & Müller 2006), that has not been analyzed.

1.5 Pcl and its role in H3-K27 trimethylation

Previous studies investigated the function of Pcl, a substoichiometric component of PRC2, in PcG repression in *Drosophila*. Nekrasov et al. 2007 found that Pcl is not required for mono- or dimethylation of H3-K27, but apparently augments the degree of H3-K27 trimethylation to levels necessary for PcG protein mediated gene repression. Levels of H3-K27 mono- or dimethylation levels seem not to be affected by Pcl knock-down (Nekrasov et al. 2007).

Pcl null mutants are embryonic lethal, and flies knocked down for this protein die in the early pupal stage (Nekrasov et al. 2007; Savla et al. 2008).

It is not yet clear whether the fact that higher levels of H3-K27 trimethylation are necessary for gene repression is due to general augmentation of H3-K27 trimethylation levels or whether specific positions of nucleosomes need to be trimethylated at H3-K27 (Nekrasov et al. 2007). Furthermore, only speculations about the role of Pcl in the Pcl-PRC2 complex exist. One possibility could be that Pcl acts as an anchor to support the recruit of PRC2 to PREs at Polycomb target sites to augment the efficiency of H3-K27 trimethylation .

1.6 Known domains of Pcl

Drosophila Pcl contains two PHD domains and a Tudor domain. It has been shown that the PHD domains bind to E(z) and mediate the interaction with PRC2 (O'Connell et al. 2001). Tudor domains are known to bind histone modifications like methylated lysine or arginine residues and are hence predestined to recruit histone modifying proteins or complexes to their target for further modifications at specific nucleosomes. Therefore, Pcl might act as a scaffold protein to recruit PRC2 to nucleosomes for efficient H3-K27 trimethylation. However, no such binding target for the Tudor domain in *Drosophila* Pcl has been found in vitro, and the function of this domain is therefore still unknown (Friberg et al. 2010). Nevertheless, it is possible that interacting combinations of domains can enable Pcl to bind histone residues, as is assumed for Pcl homologues in humans (Callebaut & Mornon 2012; Musselman et al. 2012).

1.7 Pcl and its DNA binding ability

Drosophila Pcl was recently found to have DNA-binding activity. Structural work by Jeongyoon Choi in our lab revealed that Pcl contains a winged-helix (WH) domain that binds to DNA in vitro. The Pcl WH domain binds PRE DNA sequences without any apparent sequence specificity. Analysis of the Pcl WH structure and comparison with the structure of the WH domain of FOXO4 in complex with DNA permitted to identify candidate residues in the Pcl WH domain that might be critical for DNA-binding in vitro. In vitro binding studies confirmed that mutation of these residues in the third helix and in the wing of the WH domain greatly reduce DNA-binding by the Pcl WH domain (unpublished data).

Pcl homologues can also be found in humans and here it has been postulated that the interplay between its PHD domains and a subsequent WH domain enable the protein to bind directly to DNA. It has been proposed that binding to DNA is important for recruiting histone modifying proteins to their target sites (Casanova et al. 2011). These domains have also been found in *Drosophila* Pcl, leading to the assumption that Pcl might have a similar function in *Drosophila*, like the recruitment of PRC2 to PREs.

2 Material

2.1 Buffers and solutions

Annealing buffer:	200 mM Tris-Hcl pH 8, 500 mM NaCl, 20 mM $MgCl_2$
BBT:	1% BSA and 0.1% Triton X-100 in PBS
Cross-linking solution:	50 mM Hepes pH 7.9, 1 mM EDTA pH 8, 0.5 mM EGTA pH 8, 100 mM NaCl, 1.8% Formaldehyde
Dialysis buffer (ChIP):	5% Glycerol, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 0.5 mM EGTA pH 8
Dialysis buffer (Chrom. prep.):	5% Glycerol, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 0.5 mM EGTA pH 8
LiCl buffer:	250 mM LiCl, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 0.5% NP-40, 0.5% Sodium deoxycholate
Nuclear lysis buffer:	50 mM Hepes pH 7.9, 10 mM EDTA pH 8, 0.5% N-Lauroylsarcosine, 1x Compete protein inhibitor cocktail
PBS:	3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4
PBT:	0.1% Tween20 in PBS
Ponceau S:	0.1% (w/v) Ponceau S in 5% (v/v) Acetic acid
RIPA buffer 140 mM NaCl:	140 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 1% Triton X-100, 0.1% SDS, 0.1% Sodium deoxycholate, 1 mM AEBSF, 1x Complete protein inhibitor cocktail
RIPA buffer 500 mM NaCl:	500 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8, 1% Triton X-100, 0.1% SDS, 0.1% Sodium deoxycholate, 1 mM AEBSF, 1x Complete protein inhibitor cocktail
Solution A:	10 mM Hepes pH 7.9, 10 mM EDTA pH 8, 0.5 mM EGTA pH 8, 0.25% Triton X-100

Solution B:	10 mM Hepes pH 7.9, 1 mM EDTA pH 8, 0.5 mM EGTA pH 8, 0.01% Triton X-100, 200 mM NaCl
Sonication buffer:	10 mM Hepes pH 7.9, 1 mM EDTA pH 8, 0.5 mM EGTA pH 8, 1x Complete protease inhibitors, 1 mM AEBSF, 0.5% N-Lauroylsarcosine
Stop solution:	125 mM Glycine, 0.01% Triton X-100, PBS
TBE buffer:	89 mM Tris pH 8.3, 89 mM Boric acid, 2 mM EDTA pH 8.0
TE buffer:	10 mM Tris- pH 8.3, 1 mM EDTA pH 8
Wet transfer buffer:	25 mM Tris pH 8.3, 192 mM Glycine, 0.05% SDS, 20% Methanol

2.2 Commercial material

Chromatography paper 3 MM Chr (Whatman)

Cellstar serological pipettes (Greiner bio-one)

Centrifuge tubes (TPP)

ECL Prime Western Blotting Detection Reagent (Amersham)

Fluoromount-G (Southern Biotech)

Hybond ECL Membrane 0.2 µm (Amersham)

Hyperfilm ECL High performance chemiluminescence film (Amersham)

IgG Sepharose beads (GE Healthcare)

MicroAmp 96-well Support Base (Applied Biosystems)

Microscope cover glasses 18x18 mm (Thermo scientific)

Microscope slides (Thermo scientific)

MinElute PCR Purification Kit (Qiagen)

NuPAGE MES SDS Running Buffer (Invitrogen)

NuPAGE Novex 4-12% Bis-Tris Gels (Invitrogen)

Pipette tips (Eppendorf)

PS tubes 14 mL (Greiner bio-one)

QIAprep Spin Miniprep Kit (Qiagen)

QIAquick Gel Extraction Kit (Qiagen)

Shape spreader (VWR)

Siliconized tubes (Eppendorf)

Standard RC dialysis membranes MWCO 3500 Daltons (Spectrumlabs)

Steritop 0.22 µm sterile filter (Millipore)

Tubes (Eppendorf)

2.3 Enzymes and chemicals

Acetic acid (Sigma Aldrich)

AEBSF Pefabloc (Roth)

Agarose (Sigma Aldrich)

Ampicillin (Sigma Aldrich)

BSA pulver (Sigma Aldrich)

BSA 100x solution (New England Biolabs)

DNA loading dye (6x) (Fermentas)

ECL Plus Western Blotting Detection Reagents (Amersham)

EDTA (Merck)

EGTA (Sigma Aldrich)

Ethanol 96% (Roth)

Formaldehyde 35% (Roth)

GeneRuler 1 kb DNA Ladder (Fermentas)

GeneRuler 1 kb Plus DNA Ladder (Fermentas)

Glycine (Sigma Aldrich)

HCl (Prolabo)

Hepes (Sigma Aldrich)

Igepal CA-630 (Sigma Aldrich)

Isopropanol (Sigma Aldrich)

KCl (Roth)

LB medium Difco LB Broth (BD)

LDS Sample Buffer (Invitrogen)

LiCl (Merck)

Methanol (Sigma Aldrich)

$MgCl_2$ (Merck)

N-Lauroylsarcosine 20% (Sigma Aldrich)

Na_2HPO_4 (Sigma Aldrich)

NaCl (Prolabo)

NaOH (Merck)

Nonfat dried milk powder (AppliChem)

Phusion high fidelity mastermix (Thermo)

Power Sybr Green PCR Master Mix (Applied Biosystems)

Precision Plus Protein All Blue standards (BioRad)

Protease inhibitor cocktail tablets (Roche)

Proteinase K (Roche)

Rapid DNA Ligation Kit (Roche)

Digestion enzymes (New England Biolabs)

Digestion buffers (New England Biolabs)

RNase (Roche)

SDS (Merck)

Sodium deoxycholate (Merck)

Sybr Safe DNA gel stain (Invitrogen)

Tris base (Sigma Aldrich)

Triton X-100 (Sigma Aldrich)

Tween20 (Sigma Aldrich)

2.4 Antibodies and staining solutions

Cy3 coupled α-mouse IgG (goat), affinity purified, 1:500 (Jackson ImmunoResearch)

Hoechst 33342 fluorescent molecule 50 ug/mL, 1:250 (Thermo scientific)

HRP-coupled α-rabbit IgG (donkey), affinity purified, 1:5000 (Amersham Biosciences)

α-Abd-B (mouse), monoclonal, 1:200 (from S.E. Celniker, Lawrence Berkeley National Laboratory, California, USA)

α-Esc (rabbit), , 1:5000 (from V. Pirotta, University of Geneva, Switzerland)

α-E(z) 3TAF (rabbit), crude serum, 1:7000 (Core facility, MPI, Martinsried)

α-Histone H3-K27me3 (rabbit), crude serum, 1:5000 (from T. Jenuwein, Max Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany)

α-Histone H4 (rabbit), polyclonal, 1:5000 (Abcam)

α-Pcl PHD1+PHD2+winged helix domain (rabbit), crude serum, 1:200 (Core facility, MPI, Martinsried)

α-Ubx (mouse), monoclonal, 1:30 (from R. White, University of Cambridge, UK)

2.5 *Drosophila melanogaster* strains

Controls:	w^{1118} (Jürg Müller's lab)
	yw ; UAS-GFP (Jürg Müller's lab)
Gal4 drivers	w/w ; Sp/CyO ; da-Gal4 (w^+)/da-Gal4 (w^+) (Jürg Müller's lab)
	w ; + ; tub-Gal4 (w^+)/TM6B GFP (w^+) (Jürg Müller's lab)
	w ; Sp/CyO ; tub-Gal4 (w^+)/TM2 (Jürg Müller's lab)
Pcl^{21M22} strain	yw ; F40 F42 y+ Pcl^{21M22}/SM6B (Jürg Müller's lab)
NTAP-Pcl strain	yw ; NTAP-Pcl F40 F42 y+ Pcl^{21M22}/SM6B (Jürg Müller's lab)
UAS-Pcl-RNAi strains:	Richard Jones' stocks transcribed in (Savla 2008) (gift from Richard Jones' research group, Department of Biological Sciences, Southern Methodist University, Dallas, Texas, USA)
	Stocks N°31189, N°33945, N°33946 from Bloomington *Drosophila* Stock Center, Indiana University, USA
	Stocks N°v22004, N°v22005 from Vienna *Drosophila* RNAi Center, Austria

FRT42D GFP	hs Flp122 ; F42 ubi-GFP/Cyo GFP (gift from Anne Classen's research group, Biozentrum, Martinsried)
VK33	Dmel\PBac{y+-attP-3B}VK00033 (Flybase)
Further stocks:	ywhsFlp122 ; Sp/Cyo ; TM2/TM6B (Jürg Müller's lab)
	w ; (If ; Sb)/S^T (Jürg Müller's lab)
	yw ; + ; Dr/TM6C (Jürg Müller's lab)
	w ; If/CyO (Jürg Müller's lab)

2.6 Equipment

Centrifuges:	Beckman Coulter Allegra X30R S6069
	Eppendorf centrifuge 5415D
	Heraeus Megafuge 40R
Electrophoresis:	Amersham Electrophoresis Power Supply EPS 301
	Hoefer Mighty Small Transphor
	Invitrogen XCell SureLock Mini-Cell
Microscopes:	LEICA TCS SP2 Confocal Laser Scanning Microscope (Confocal microscope)
	Zeiss Axio Scope.A1 (Fluorescence microscope)
	Zeiss SteREO Discovery.V8 (Fluorescence microscope)
	Zeiss SV11 Stereomicroscope

PCR machines:	Applied Biosystems StepOnePlus Real-Time PCR Systems
	BioRad C1000 Thermal CyclerAgitator
Further equipment:	Amersham Hypercassette
	Diagenode Bioruptor Next Gen
	Eppendorf Multipette Stream
	Eppendorf Research plus pipettes
	Eppendorf Thermomixer Comfort
	Peqlab Nanodrop 2000C
	Ika Ikamag RCT magnetic stirrer
	Inolab 720 pH meter
	Integra Biosciences Pipetboy acu
	Rotating wheel
	Sartorius TE 1502S scales
	Scientific Industries Vortex Genie 2

3 Methods

The composition of buffers and solutions mentioned in this chapter are listed in 2.1. Sources of commercial material, enzymes, chemicals, DNA, antibodies and staining solutions are listed in 2.2-2.4. The *Drosophila* stocks are described in 2.5 and a description of the equipment is given in 2.6.

3.1 Obtention of Pcl knock-down larvae

Virgin females expressing the driver da-Gal4 or tub-Gal4 were selected and crossed with males bearing the UAS-Pcl-RNAi construct. The flies were kept at 25°C and vials with yeasted agar were changed roughly every second day to collect offspring of the line da-Gal4/UAS-Pcl-RNAi or tub-Gal4/UAS-Pcl-RNAi. To visualize the spatial and temporal expression enhanced by the UAS, an identical cross was performed in parallel to the aforementioned cross, except that males of the line UAS-GFP were used.

3.2 Obtention of Pcl knock-out clones in imaginal discs of wandering third instar larvae

hsFlp122 ; F42 ubi-GFP/CyO virgins were crossed with yw. F42 y^+ Pcl^{21M22}/SM6B males and the offspring were heat-shocked in the first instar larval stage for 1 h in a water bath at 37°C to turn on expression of Flp recombinase; they were then kept at 25°C for 72 h. Wandering third instar larvae were selected and prepared for immunostaining as described in 3.6.

3.3 Preparation of sonicated imaginal disc extracts

100 wandering third instar larvae of each Pcl Knock-down cross and 100 wandering third instar larvae of the strain w^{1118} were selected and dissected in PBS buffer kept on ice. After isolating brain and imaginal discs of wing, haltere, 1st, second, and third leg, eye and antenna, 1 mL of PBS with 0.1% NP40 in a final concentration was added to the discs to facilitate the transfer of them to siliconized eppendorf tubes. Siliconized tips were used for this transfer. The discs were centrifuged for 30 s at room temperature at maximal speed and the supernatant was discarded. LDS buffer was then added to a final concentration of 1x LDS and the discs were stored at -20°C. Then, the discs were sonicated for 8 cycles with 30 s of sonication and 30 s break in a total volume of 100 µL 1x LDS and then heated up to 80°C for approximately 3 min. To improve the fragmentation of residual chromatin, the sample was sonicated again and heated up to 80°C for approximately 3 min. The sonicated extract was centrifuged for 3 min at maximum speed and the supernatant was transferred into a new tube and analyzed by gel electrophoresis.

3.4 SDS gel electrophoresis

10 µL of sonicated extract of each sample (i.e. equivalent of 10 larvae, see 3.3) and 15 µL protein standard were loaded and separated on a 4-12% precast gradient gel for 50 min at 150 volts in MES buffer.

3.5 Western blot and antibody detection

After electrophoresis, the gels and blotting membranes were equilibrated for about 5 min in wet transfer buffer and then were blotted on the membranes for 2 h at 90 volts using wet transfer buffer. After the blot, the membranes were stained with Ponceau S to confirm that the proteins had been transferred onto the membrane. The membrane was rinsed with H_2O to remove residual staining solution, and blocked in 5% nonfat dried milk powder in PBT overnight on a shaker at 4°C. The membranes were incubated with the corresponding primary antibodies

diluted in 5% nonfat dried milk powder in PBT overnight on a shaker at 4°C. The antibodies were used according to the dilutions listed in 2.4. Where different antibodies were used for detection, membranes were cut into strips before incubation with primary antibodies. Afterwards the membranes were washed in PBT for 30 min on a shaker at room temperature. Then they were incubated with the corresponding HRP-labeled secondary antibodies diluted 1:5000 in milk solution for 2-3 h on a shaker at room temperature. The membranes were washed again with PBT for 30 min on a shaker at room temperature and covered with 1 mL ECL Plus Western Blotting Detection Reagents in order to start the HRP catalyzed analytical reaction. Signal detection was accomplished on a film after different exposure times.

3.6 Immunostaining of imaginal discs

Larval cuticles of wandering third instar larvae were obtained by inverting anterior halves of cuticles and subsequent removal of the digestive system and fat tissue. All of these steps were performed in PBS kept on ice. The cuticles were transferred to siliconized eppendorf tubes through the use of siliconized tips; they were then fixed with 4% formaldehyde in PBS on a shaker for 20 min at room temperature. The cuticles were washed twice in PBT and then four times in BBT for 1h at room temperature for blocking. After washing, the cuticles were incubated with 40 µL of the corresponding primary antibody diluted in BBT overnight on a rotating wheel at 4°C. The cuticles were washed six times in BBT on a shaker and then incubated with 40 µL of the corresponding secondary antibody diluted 1:500 in BBT and 1 µL Hoechst overnight on a rotating wheel and protected from light at 4°C. After incubation, the cuticles were washed twice in BBT and then four times in PBT for 1h on a shaker at room temperature. Afterwards the discs of interest were dissected in PBT and mounted on a microscope slide in 12 µL Flouromount-G under a 18x18x1 mm coverslip.

3.7 Preparation of larval chromatin for Chromatin Immunoprecipitation (ChIP)

Larval cuticles of 300 wandering third instar larvae were obtained as described in 3.6. The cuticles were transferred to siliconized eppendorf tubes and then cross-linked in cross-linking solution on a shaker at room temperature for 20 min. The cross-linking solution was changed five times. Afterwards the cross-linking solution was removed and the cross-linking reaction was stopped by washing the cuticles in stop solution (125 mM Glycine) on a shaker at room temperature for 10 min; the cross-linking solution was changed five times. The washing procedure was repeated first using solution A and then using solution B to slowly increase the salt concentration to denature the chromatin for subsequent fragmentation by sonication. The cuticles were stored at 4°C until dissection of the imaginal discs.

Brain and imaginal discs of eye, antenna, first, second and third leg, wing and haltere were dissected on ice in solution B and transferred to siliconized eppendorf tubes through the use of siliconized tips. At this step, the discs were separated into three aliquots to set up three independent experiments. Solution B was removed from the discs and sonication buffer was added to a final volume of 2 mL; the discs were transferred to 15 mL polystyrene tubes kept on ice. The discs were sonicated in 12 cycles with 30 s of sonication and 30 s breaks in between with the settings of the Biorupter on “high”. The falcons were cooled on ice after about 6 cycles to avoid warming up the samples. After sonication N-Lauroylsarcosine was added to a final concentration of 0.5% and the samples were rotated for 10 min at 4°C. The sonicated discs were transferred into siliconized eppendorf tubes and centrifuged for 10 min at 4°C at maximum speed to pellet insolute debris.

A dialysis membrane was washed in ddH_2O and equilibrated in dialysis buffer. The discs were dialyzed in dialysis buffer at 4°C with rotation overnight and dialysis buffer was changed twice after 2h and 4h during dialysis to decrease the salt concentration again. The chromatin was transferred to non-siliconized eppendorf tubes and aliquots for the immunoprecipitation and for input were snap frozen in liquid nitrogen for storage at -80°C.

3.8 DNA purification for quantitative polymerase chain reactions

2 µL RNase were added to the chromatin in 100 µL TE buffer and the samples were incubated on a shaker for 30 min at 37 °C at 750 rpm. Proteinase K was added up to a final concentration of 0.5mg/mL and SDS up to a final concentration of 0.5% was supplied to ameliorate protein degradation by unfolding the substrates. The samples were incubated on a shaker for about 9-13 h at 37°C at 750 rpm for protein digestion and for 6 h at 65°C at 750 rpm to reverse the formaldehyde cross-links. The DNA was purified using the Minelute purification kit according to a procedure adopted from the company's protocol. The samples were supplied with 600 µL PB buffer and 10 µL 3 M sodium acetate pH 5.2 and transferred to MinElute columns. After centrifuging for 1 min at maximum speed at room temperature, the column was loaded with 750 µL PE buffer, incubated for 5 min at room temperature and centrifuged for 1 min at maximum speed at room temperature to wash the column. The latter washing step was repeated once and the columns were finally centrifuged for 1 min at maximum speed at room temperature to discard residual ethanol. DNA was eluted from the columns with elution buffer warmed up to 65°C; it was then incubated for 1 min at room temperature and centrifuged for 1 min at maximum speed at room temperature. The elution volumes for samples prepared for qPCR were 500 µL and for samples prepared for gel electrophoresis 10 µL.

To determine the length of obtained chromatin fragments 80 µL aliquots of each independent chromatin were purified and separated on a 1% agarose gel in TBE buffer.

3.9 Quantitative real-time polymerase chain reaction

For DNA quantification, samples with final volumes of 25 µL were set up containing 10 µL of each sample chromatin, primers flanking a genomic fragment (PRE or gene region) of interest in final concentrations of 0.5 µM forward and 0.5 µM reverse, and 1x Power Sybr Green PCR Master Mix. Targeted genomic fragments were quantified by measuring the increase of fluorescence after intercalation of supplied Sybr Green into double stranded PCR product. The thermocycling program was set as follows.

	2 min	50°C
	10 min	95°C
40 cycles:	15 s	95°C
	1 min	60°C
	30 s	95°C
	15 s	60°C
final		4°C

The amounts of DNA present in the input and IP samples were determined relative to a standard curve constructed with 5 serial four-time dilutions of a standard DNA (i.e. the most concentrated standard was 256x more concentrated than the most diluted one), designed to cross the fluorescence threshold after 22, 24, 26, 28 and 30 cycles, respectively. They were named S22-S30.

Inputs were prepared from 30 µL aliquots of each chromatin: The amount of each chromatin used for each IP was determined by comparing its DNA content to that of chromatins from previous experiments in which its amounts had been optimized.

3.10 Chromatin Immunoprecipitation with PAS beads

Chromatin from third instar larvae was prepared as described in 3.7. The appropriate chromatin aliquots were thawed on ice and adjusted to final concentrations of 1% Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS, 140 mM NaCl, 1x Complete protein inhibitor cocktail and 1 mM AEBSF up to a volume of 1 mL. IgG Sepharose beads were washed three times with RIPA buffer 140 mM NaCl, each time while incubating for 10 min at 4C° and then spinning down the beads by centrifuging for 2 min at 4000 rpm at 4°C to remove the supernatant. The concentration of beads was adjusted with RIPA buffer 140 mM NaCl up to 50% (v/v) and 40 µL beads was added to the chromatin samples.

The beads were washed once in RIPA buffer 140 mM NaCl, four times in RIPA buffer 500 mM NaCl, once in LiCl buffer and finally twice in TE buffer, each time while incubating for 10 min at 4C° and then spinning down the beads by centrifuging for 2 min at 4000 rpm at 4°C to remove the supernatant. After washing the beads, the chromatin bound to the beads was purified as described in 3.8.

The enrichment of a genomic region after IP was expressed as:

$$\%\ of\ input\ chromatin = 100x\ \frac{IP\ value}{input\ value\ of\ chromatin\ used\ per\ IP}$$

Values for IP and input chromatin were measured in parallel PCR reactions on the same 96-well plate and relative to the standard curve to ensure comparable fluorescence signals from equal reaction conditions. A mean value was determined after calculating the enrichment from three independent experiments.

3.11 Plasmid transformation of competent bacteria cells and plasmid purification

For transformation, 50 µL of $CaCl_2$ treated competent cells from the corresponding bacterial strain were thawed on ice and supplied with about 200-300 ng of vector. After 20 min of incubation on ice, the cells were heat-shocked for 90s at 42°C and afterwards left on ice for 10 min. Afterwards 200 µL LB medium without antibiotic was added and the cells were shaken for about 1 h at 37°C at 300 rpm. The cells were plated on LB containing agar plates with the appropriate antibiotic to select for transformants and incubated overnight at 37°C. For plasmid preparations, colonies were picked to inoculate 5 mL of LB medium supplied with the corresponding antibiotic and shaken overnight at 37°C at about 225 rpm. Plasmids were then isolated using the QIAprep Spin Miniprep Kit following the manufacturer's instructions.

3.12 Synthesis of an HA-Flag-Flag tag

The HA-Flag-Flag tag to precipitate Pcl in the ChIP assays was synthesized through the assembly of 3 5' phosphorylated and HPLC purified DNA oligos constituted of the HA tag part and two Flag tag parts. Those fragments were first annealed separately as corresponding forward and reverse oligonucleotides and then used in the appropriate amounts in a ligation assay, where they were ligated with each other but also into the respective vector as the fragments were synthesized in such a way that they consisted of overlapping regions that reconstructed the complete HA-Flag-Flag tag in the correct order flanked by 5' overhangs that were compatible with the digested ends of the linearized vector.

The annealing reactions were accomplished in 50 µL reactions in annealing buffer with 45 µM forward oligonucleotides and 45 µM reverse oligonucleotides respectively, according to the following procedure in a PCR machine. The oligonucleotides were first incubated for 3 min at 94°C and then cooled down stepwise from 90°C to 25°C, reducing the temperature by 0.5°C every 15 s.

3.13 Digestion and ligation for vector cloning

For cloning experiments, about 10 µg of plasmid were digested by about 50 units of the corresponding digestion enzyme supplied with the corresponding buffer and optionally BSA in a final concentration of 100 µg/mL in 50 µL of total volume. To ensure complete digestion, plasmids were digested overnight at room temperature and the desired digestion product was either purified on a preparative 1% agarose gel in TBE and isolated using the QIAquick Gel Extraction Kit or purified using the MinElute PCR Purification Kit to remove inserts up to a size of 200 bp. Ligation reactions were accomplished in 1:3 vector insert ratios using the Rapid DNA Ligation Kit following the manufacturer's instructions.

3.14 Introduction of point mutations by PCR

Point mutations were introduced into the corresponding plasmids by PCR with primers that included the corresponding mutation flanked by regions with generally 15-30 base pairs without mismatches to ensure correct annealing to the template. About 20 ng of template vector was used and forward and reverse primers were added in final concentrations of 0.2 µM each. The reaction was accomplished with Phusion high fidelity mastermix in a linear amplification during the following steps in a total volume of 50 µL.

	30 s	98°C
25 cycles:	10 s	98°C
	30 s	58°C
	9 min	68°C
	10 min	68°C
final		12°C

Afterwards, 1 µL DpnI was added and the amplification product was incubated for 1h at 37°C to digest the methylated and not mutated template vector. This was then transformed into competent DH5α bacteria cells or competent JM110 bacteria cells as described in 3.11.

3.15 Cloning of HA-Flag-Flag and Pcl into pUAST attB

The HA-Flag-Flag tag was obtained as described in 3.12 with overhangs corresponding to EcoRI digestion sites at the 5' end and NotI digestion sites at the 3' end.

pUAST attB (Bischof et al. 2007) was digested with EcoRI and NotI and purified using the QIAprep Spin Miniprep Kit, and the HA-Flag-Flag tag was ligated into pUAST attB as described in 3.13.

Pcl was obtained from the plasmid pFastBac-dual (Invitrogen), which had been modified by introduction of an N-terminally tagged Pcl transgene. This transgene had a NotI digestion site at the 5' end. A XbaI digestion site at the 3' end as well as mutations into DNA binding regions were introduced as described in 3.14.

pFastBac-dual-HA-Pcl without point mutated DNA binding sites or after introduction of point mutations into the DNA binding helix and turn was transformed into JM110 bacteria cells as described in 3.11 to avoid dam methylation of the XbaI digestion site. This step was important to allow subsequent digestion by XbaI, which is a dam methylation sensitive enzyme. After isolating the plasmid, the Pcl sequence was cut out by digestion with NotI and XbaI, purified on a gel and then ligated into the digested and purified pUAST attB-HA-2xFlag vector as described in 3.13.

3.16 Verification of cloning constructs

The results after each cloning step were verified by digestion of 0.5 µg of plasmid by about 10 units of the corresponding digestion enzyme; these enzymes were supplied with the corresponding NE buffer and optional BSA in a final concentration of 100 µg/mL in 10 µL of total volume. Digestion was performed for 1 h at 37°C and the band pattern was analysed on a 1% agarose gel in TBE. Sequences changed by mutagenesis reactions were verified using the in house sequencing service or MWG.

3.17 Introduction of point-mutated HA-Flag-Flag-Pcl constructs into the fly genome

The target vector pUAST attB (Bischof et al. 2007) including the tagged Pcl construct was injected into embryos of the VK33 strain. This strain bears genomic attP recombination sites on the third chromosome and a ΦC31 integrase on the X chromosome. The pUAST attB vector contained an attB recombination site downstream of the Pcl construct. After injection of the pUAST attB plasmid into the pole cells of the embryo (i.e. the germline primordial cells),

recombination was accomplished by the ΦC31 integrase between the attB and attP sites (Groth et al. 2004). To check if the Pcl gene had been integrated into germ line cells, males of these injected VK33 were crossed with females of the strain yw ; + ; Dr/TM6C. Transgenic offspring were identified thanks to the eye colour because the VK33 strain bears a mutation in the *white* gene. This mutation turns their eye colour white because of a defect in the normal red pigmentation production. However, the injected pUAST attB plasmid was marked by the *white*$^{+}$ marker, which rescues the *white*$^{-}$ VK33 strain, and turns their eyes red. Offspring of VK33 flies in which the transgene has been integrated into the germ line are therefore *white*$^{+}$ and have red eyes. Males of the transgenic offspring were crossed again with females of the strain yw ; + ; Dr/TM6C. Offspring of this stock was screened for transgenic flies with TM6C as a balancer and kept as a stock. An overview of the accomplished crossing steps is shown in 11.5 A.

3.18 Obtention of cell clones homozygous for point-mutated HA-Flag-Flag-Pcl constructs lacking endogenous Pcl in larval imaginal discs

Transgenic males from the stock obtained in 3.17 were crossed further in order to obtain a final stock with the protein negative Pcl allele on the second chromosome distal to a F42 recombination site and the UAS-Pcl construct with the mentioned mutations inserted onto the third chromosome into the attP landing site. In parallel, another stock has been created, which bears the heat-shock induced recombinase Flp122 on the first chromosome, ubiquitously expressed GFP distal to a F42 recombination site on the second chromosome, and a Gal4 driver on the third chromosome. The Gal4 driver is either controlled by a daughterless or by a tubulin promotor. An overview of the crossing steps is shown in 11.5 B and C.

The created stocks were used to obtain Flp122 ; F42 Pcl^{21M22}/F42 ubi-GFP ; UAS-Pcl /Gal4 third instar larvae. The larvae were heat-shocked for 1 h at 37°C in a water bath to induce Flp122 mediated recombination in order to obtain cell clones lacking endogenous Pcl in larval imaginal discs of HA-Flag-Flag-Pcl expressing animals. The cell clones were analyzed by immunostaining as described in 3.6 and identified by the lack of GFP expression.

4 Results

4.1 Effects of Pcl knock-down on PcG protein mediated gene repression

To analyze the role of the PcG protein Pcl, I used RNA interference and crossed males expressing a Pcl-RNAi construct under the control of a UAS_{Gal4} promoter with female virgins expressing the transcription factor Gal4 to induce expression of the Pcl double stranded RNA. Offspring of these so-called responder and driver stocks are expected to have reduced levels of Pcl protein in tissues where Gal4 is expressed (reviewed in Duffy 2002). I performed immunostainings to analyze whether and how expression of Polycomb target genes was affected. Imaginal discs of wandering third instar larvae were isolated and analyzed to reveal the efficiency of Pcl protein knock-down. This was done by western blotting with an antibody against Pcl protein and antibodies against other PcG proteins as control and I also monitored levels of the H3-K27me3 histone modification.

In these experiments, Gal4 was expressed under a daughterless promoter or a tubulin promoter. In flies, tubulin is ubiquitously expressed (O'Donnell et al. 1994); Pcl should therefore be knocked down in all tissues. To visualize, where the Pcl-RNAi construct is expressed in the offspring of the da-Gal4 driven system, daughterless-controlled Gal4 expression was traced back by crossing virgins homozygous for da-Gal4 with males homozygous for UAS-GFP. Detection of GFP in specific tissues therefore demonstrates that the daughterless promoter is active. Figure 3 shows imaginal discs isolated from wandering third instar larvae, obtained from the aforementioned cross. GFP expression can be detected in the brain, in the optic globes, and in the imaginal discs of wing, eye, antenna, and first, second, and third leg. From these results it can be assumed that Pcl-RNAi knock-down takes place in the same tissues in animals containing da-Gal4 and the UAS-Pcl-RNAi constructs.

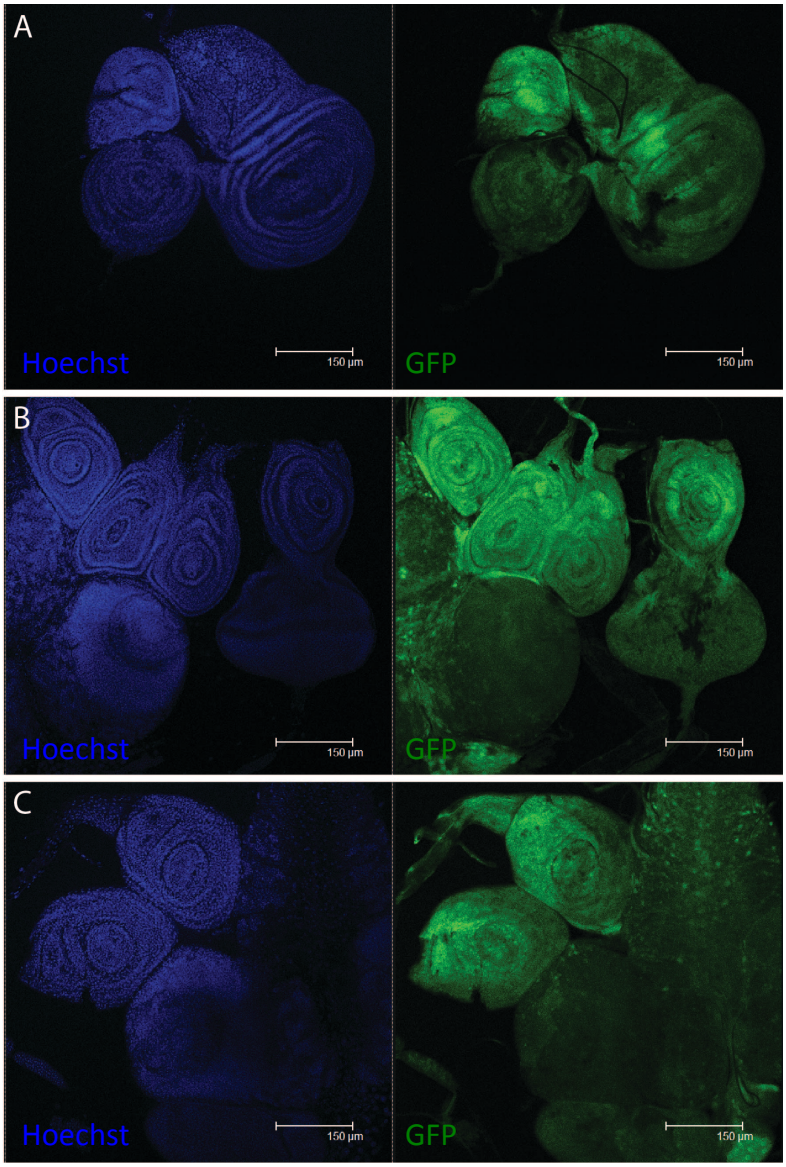

Figure 3: Imaginal discs from wandering third instar larvae obtained from the cross of virgins homozygous for da-Gal4 with males homozygous for UAS-GFP. GFP expression is used to identify tissues where the daughterless promoter is active and activates genes, which are controlled by the UAS. DNA was stained with Hoechst (blue). GFP (green) is expressed in imaginal discs of wing, third leg and haltere (A), in imaginal discs of eye and antenna (B), and in imaginal discs of second and third leg (C). To a lesser extent, GFP expression can also be detected in the nervous system and optic globules (C). Scale bars represent 150 µm.

To knock-down Pcl, 13 different responder strains bearing the Pcl-RNAi transgene were tested in crosses with flies expressing Gal4 under a daughterless or tubulin promoter. Detailed information of the stocks and description of the offspring is shown in Table 1 (see 11.5 in supplementary).

Knock-down of Pcl by Pcl-RNAi construct pWIZ-Pcl has been reported to be lethal in the early pupal stage (Savla et al. 2008). However, I found that most of the animals carrying this construct developed into the late pupal stage and in some cases even hatched, suggesting that Pcl knock-down was not efficient. However, some of the male offspring showed extra sex combs on the second legs, which is known to be a Polycomb phenotype (Kennison 1995).

Four of the stocks listed in Table 1 (marked in bold) were selected for further investigation to quantify the efficiency of Pcl knock-down. This selection has been made due to the fact that the offspring of these stocks obtained in the previously mentioned crosses show the strongest phenotype.

One approach to quantify the efficiency of Pcl knock-down is analyze expression of Polycomb target genes, such as the HOX genes *Ubx* or *Abd-B*, which should no longer be repressed upon depletion of Pcl protein.

Figure 4 and Figure 5 show the results from immunostainings of imaginal discs from third instar larvae, which were knocked down for Pcl. The larvae were obtained from crosses with da-Gal4 as a driver and the UAS-Pcl-RNAi stocks N°5, N°6, N°12 and N°13 (Supplementary, Table 1). As a control, expression of Ubx and Abd-B was analyzed in w^{1118} larvae, which should show normal Hox gene expression. Here, Ubx was detected in imaginal discs of third leg and haltere, but not in wing imaginal discs (Figure 4, "control"). Abd-B has been detected neither in imaginal discs of third leg or haltere nor in wing imaginal discs (Figure 5, "control").

These results are consistent with previous results where it has been shown that the *Ubx* gene is repressed in wing imaginal discs of third instar larvae but is expressed in third leg and haltere imaginal discs. Furthermore, the *Abd-B* gene is repressed in wing, third leg and haltere imaginal discs (Brower 1987; Beuchle et al. 2001). In contrast, in wing imaginal discs of larvae, which were knocked down for Pcl, Ubx misexpression has been detected after crossing UAS-Pcl-RNAi stocks N°12 and N°13 with da-Gal4 expressing virgins. However, no misexpression was detected in imaginal discs of larvae obtained with crosses performed with the UAS-Pcl-RNAi stocks N°5 and N°6 (Figure 4). This finding leads to the assumption that Pcl knock-down was not as efficient as in the crosses performed with the UAS-Pcl-RNAi stocks N°12 and N°13. This statement is supported by the detection of Abd-B misexpression, which seems to be more severe in imaginal discs of larvae obtained with the UAS-Pcl-RNAi stocks N°12 and N°13 than with the stocks N°5 and N°6 (Figure 5).

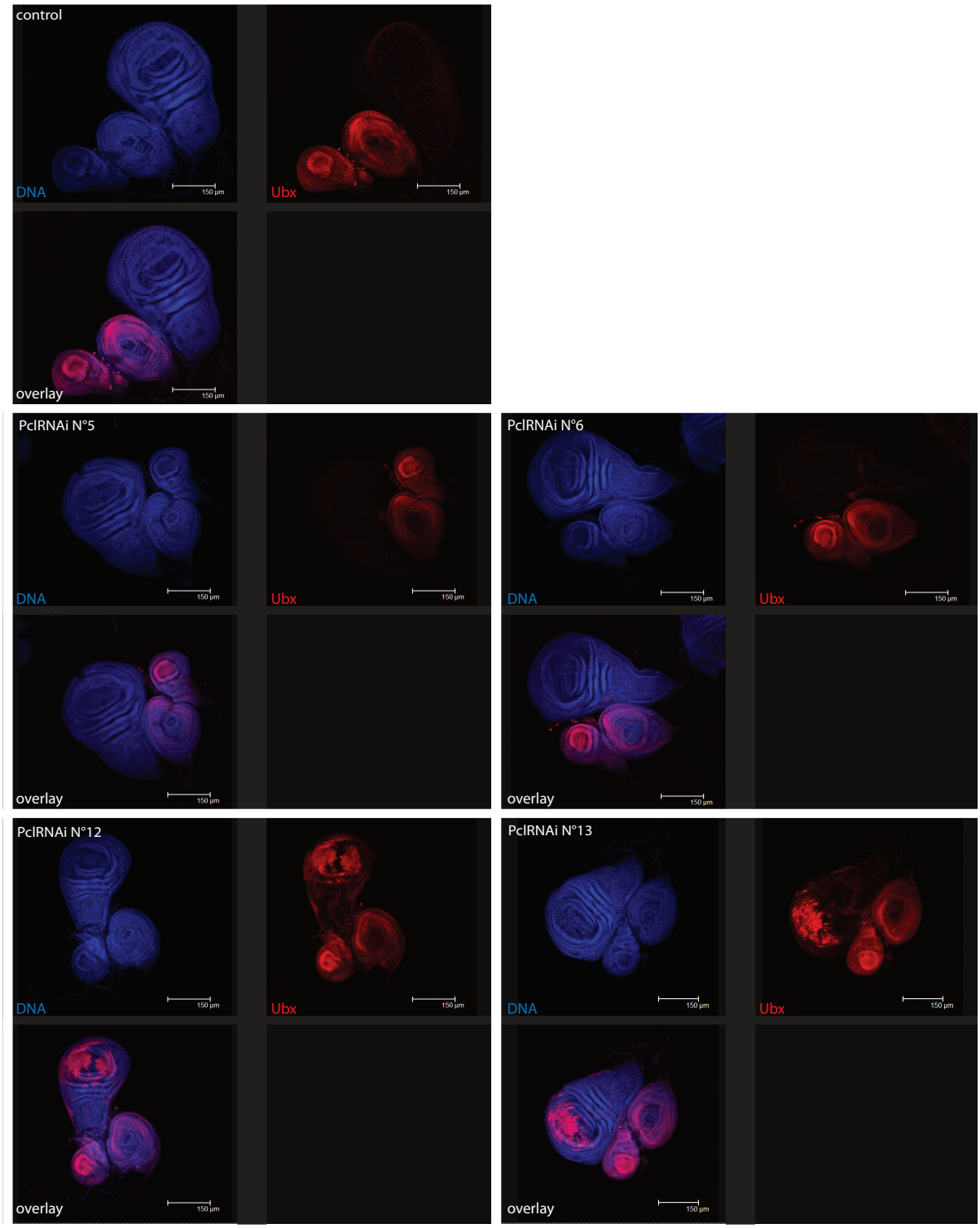

Figure 4: Imaginal discs of third instar larvae from crosses obtained with da-Gal4 virgins and males of UAS-Pcl-RNAi stocks N°5, N°6, N°12 and N°13. DNA was stained with Hoechst (blue) and a-Ubx antibodies were used to detect Ubx expression (red). Overlays show the co-localization of both signals. w^{1118} larvae served as a control. Scale bars represent 150 µm.

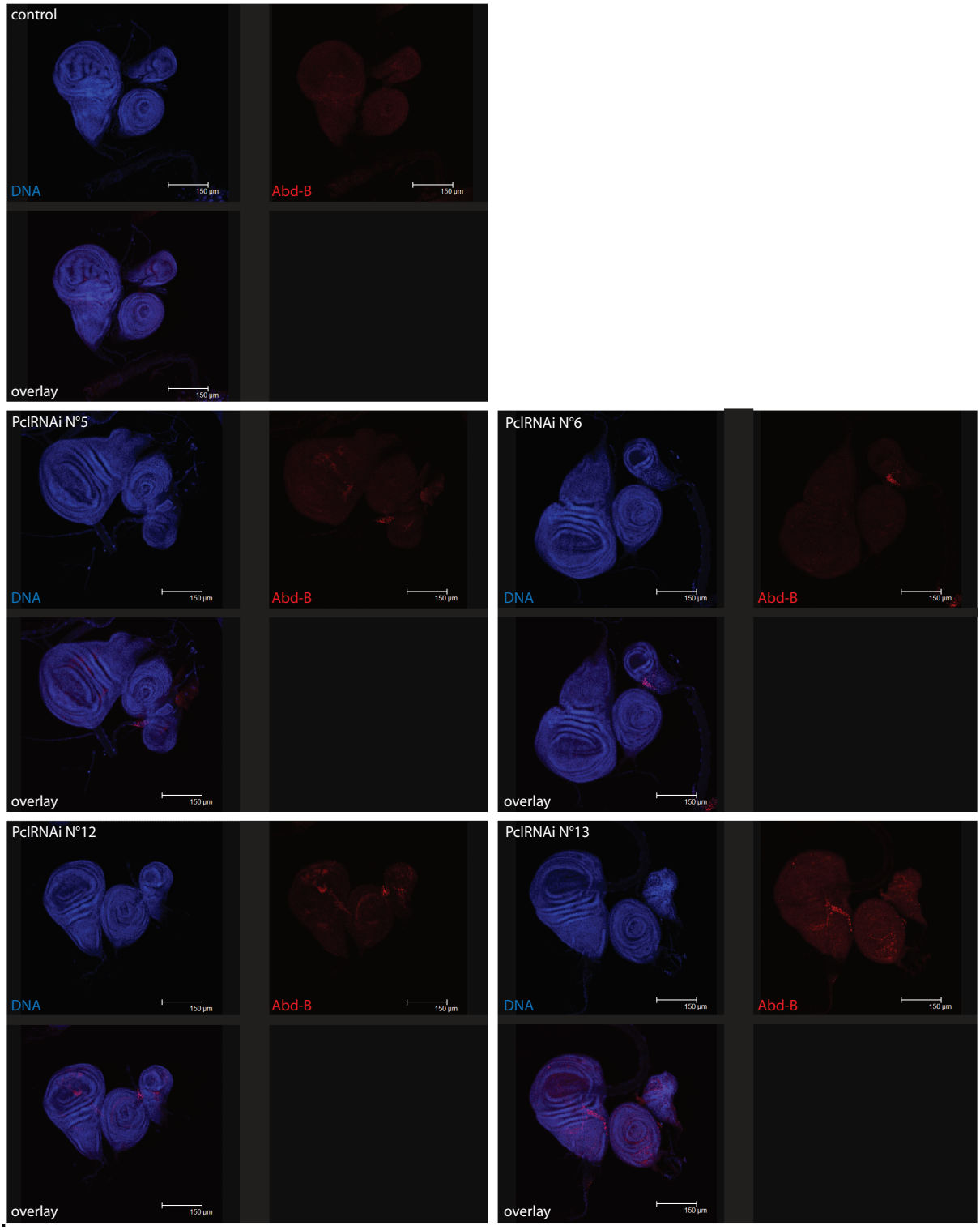

Figure 5: Imaginal discs of third instar larvae from crosses obtained with da-Gal4 virgins and males of UAS-Pcl-RNAi stocks N°5, N°6, N°12 and N°13. DNA was stained with Hoechst (blue) and a-Abd-B antibodies were used to detect Abd-B expression (red). Overlays show the co-localization of both signals. w1118 larvae served as a control. Scale bars represent 150 µm.

The misexpression of Polycomb target genes in imaginal discs after Pcl-RNAi knock-down confirms that Pcl is critical for efficient Polycomb mediated repression in imaginal discs of third instar larvae. Furthermore, the different knock-down efficiencies achieved by the crosses previously discussed showed that a certain amount of Pcl seems to be required for efficient gene repression. This could be explained by the findings from human Pcl homologues, which anchor PRC2 to Polycomb target genes (Casanova et al. 2011). If this is also the case for *Drosophila* Pcl, repression could only take place when a distinct amount of Pcl is available to recruit PRC2 to the DNA to mediate its function (i.e. recruitment of PRC1 for the modification of chromatin to achieve gene repression). However, other modes of operations cannot be excluded, like stabilizing PRC2 as a complex or accelerating its formation. Furthermore, how the increase of H3-K27 trimethylation is triggered by Pcl abundance (Nekrasov et al. 2007) cannot be explained yet.

The knock-down crosses of stocks N°12 and N°13 were repeated with virgins, which expressed Gal4 under the tubulin promoter. Immunostainings of imaginal discs from knock-down larvae were performed in order to see, if Gal4 expression can be increased to augment misexpression of Polycomb target genes. The results are shown in Figure 6 (Ubx) and in Figure 7 (Abd-B).

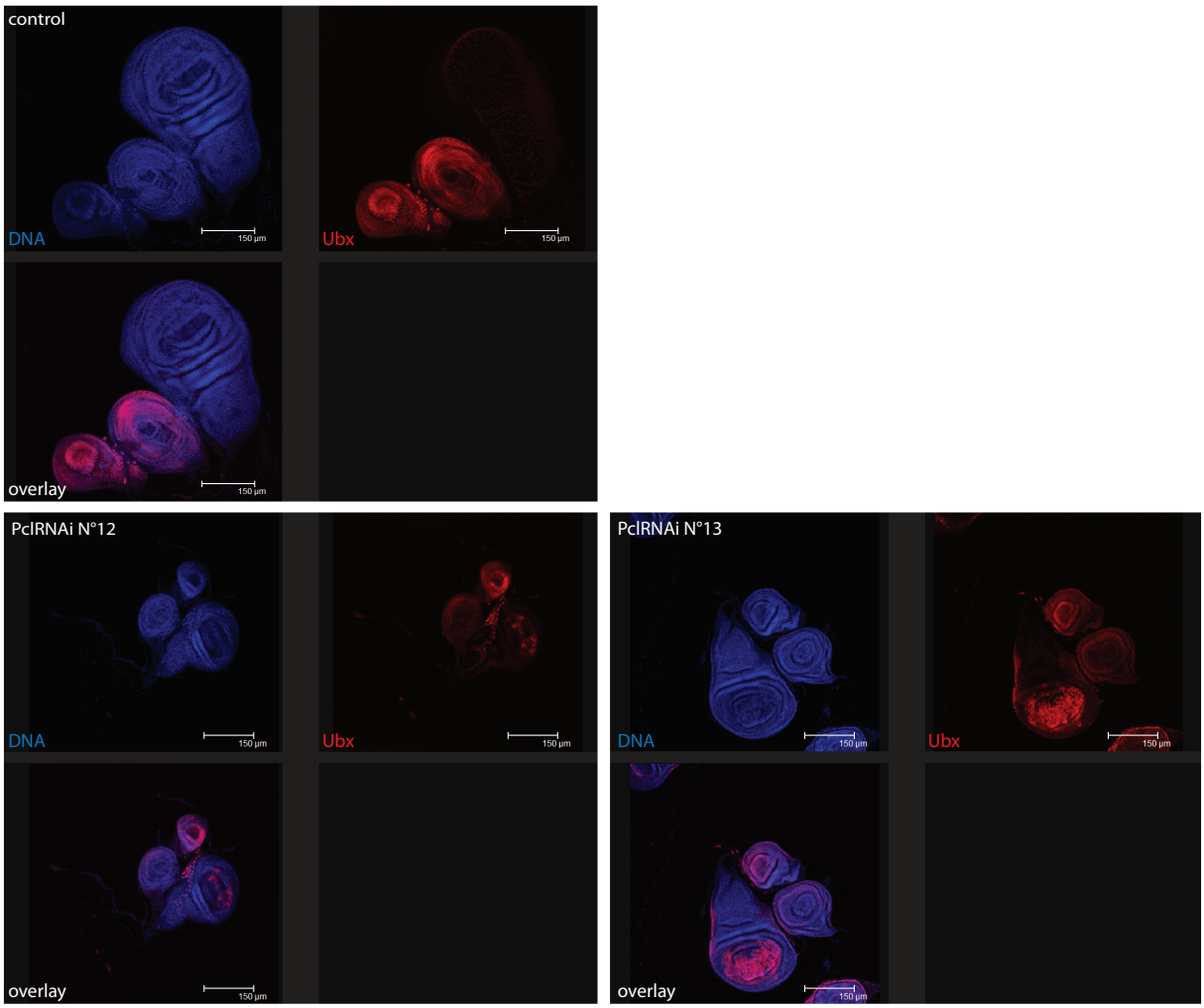

Figure 6: Imaginal discs of third instar larvae from crosses obtained with tub-Gal4 virgins and males of UAS-Pcl-RNAi stocks N°12 and N°13. DNA was stained with Hoechst (blue) and a-Ubx antibodies were used to detect Ubx expression (red). Overlays show the co-localization of both signals. w1118 larvae served as a control. Scale bars represent 150 µm.

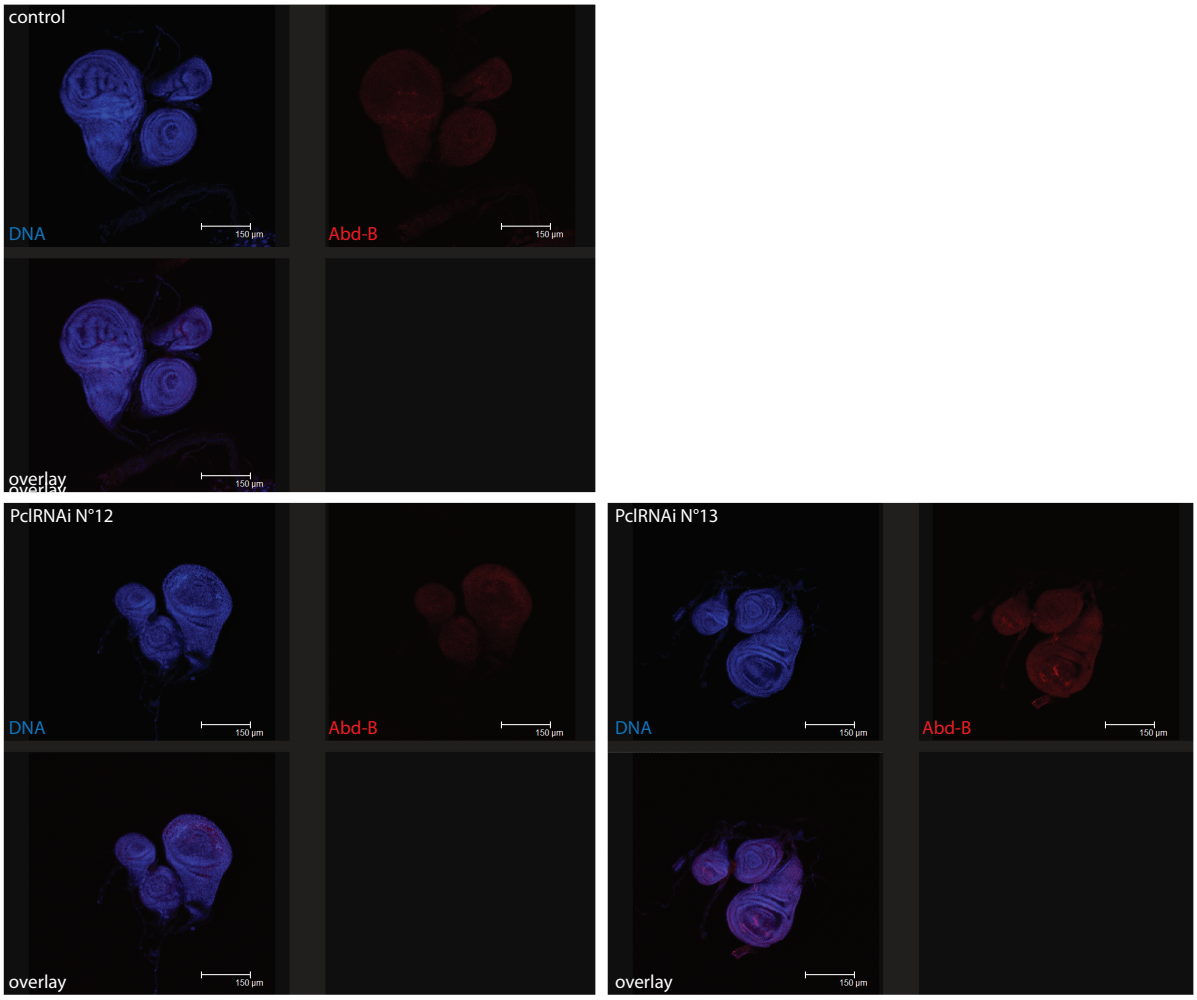

Figure 7: Imaginal discs of third instar larvae from crosses obtained with tub-Gal4 virgins and males of UAS-Pcl-RNAi stocks N°12 and N°13. DNA was stained with Hoechst (blue) and a-Abd-B antibodies were used to detect Abd-B expression (red). Overlays show the co-localization of both signals. w1118 larvae served as a control. Scale bars represent 150 µm.

Again, misexpression of Ubx can be detected in wing imaginal discs compared to the control larvae (Figure 6), but does not seem to be more severe than in knock-down discs from larvae obtained from crosses using the da-Gal4 driver. Wing imaginal discs from larvae after crossing tub-Gal4 virgins with males from stock N°12 even seem to have less Ubx misexpression than those from larvae after crossing da-Gal4 virgins.

These observations are strengthened by the results from the detection of Abd-B misexpression (Figure 7), which seems to be less severe in crosses obtained from tub-Gal4 as a driver, than from those obtained from da-Gal4 crosses.

Another approach to quantify the efficiency of Pcl knock-down is to compare amounts of Pcl by western blot on imaginal disc extracts, obtained from knock-down larvae after crossing da-Gal4

virgins with the Pcl-RNAi strains N°12 and N°13; these larvae showed the strongest effects (Figure 5-Figure 7). Brain and imaginal discs of wing, haltere, 1st, second, and third leg, eye and antenna were isolated as the daughterless promoter has been found to be active in those tissues, leading to Pcl knock-down in those tissues. The extract was obtained as described in 3.3 and diluted twice and four times. The non-diluted and the diluted samples were loaded to quantify the decrease of Pcl after detection on a membrane. Histone H4 abundance should not be affected upon Pcl knock-down and served as a loading control. Figure 8 shows the signals after detection of Pcl, the PRC2 components Esc and E(z) and trimethylated Histone H3-K27.

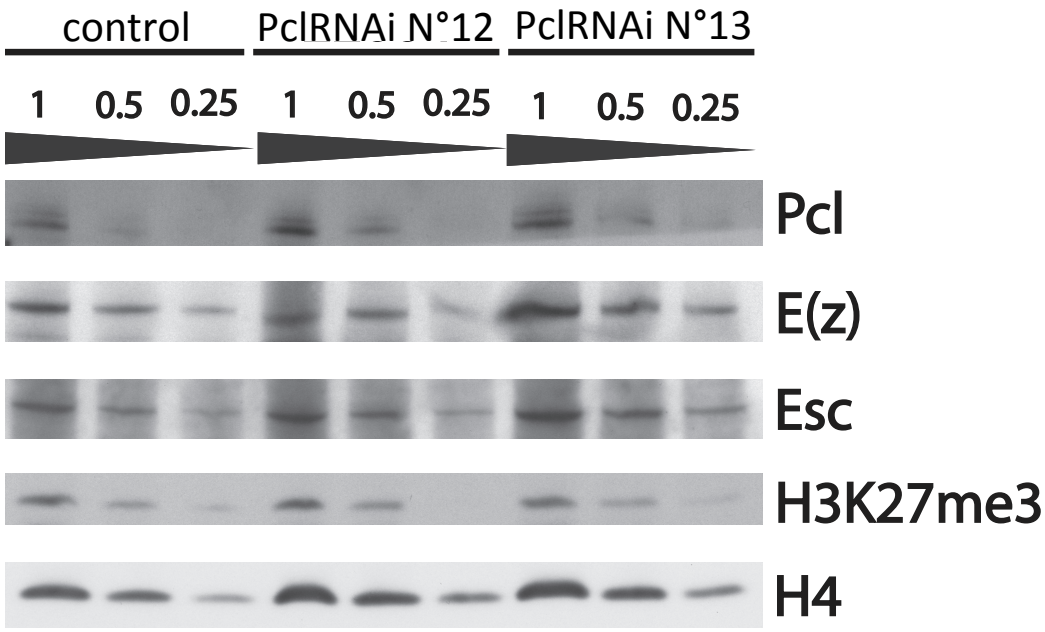

Figure 8: Western blot on sonicated extract of imaginal discs from third instar larvae after crossing da-Gal4 virgins with males of the strains N°12 and N°13. w^{1118} larvae served as a control and Histone H4 detection was used as a loading control.

Strikingly, I was unable to detect a significant decrease of Pcl protein in the analyzed tissues. Similarly, H3-K27me3 levels appear undiminished. These results are surprising, because Polycomb repression of Ubx and Abd-B was lost in wing imaginal discs in these animals.

The same results were obtained from imaginal disc extract of larvae, which where obtained after crossing da-Gal4 virgins with males of the strain N°5 and N°6 (data not shown).

It is possible, that the decrease of Pcl and H3-K27me3 are too small to be detected on a blot but sufficient to cause misexpression of target genes. This could be due to the fact that the tissues used for the analysis of protein levels also included tissues with lower activity of the

daughterless promoter, such as the nervous system, were used. As judged by the GFP expression levels (Figure 3), Pcl levels would be expected to be less diminished in these tissues.

Another possibility could be that antibody detection was not specific. The antibody, which has been used to detect Pcl in the western blot, was raised against an epitope containing the PHD finger and WH domain of Pcl. At present, we cannot exclude that the antibody cross-reacts with another protein that runs at the same size like Pcl on an SDS polyacrylamide gel. To rule out this possibility, a western blot on extracts from Pcl protein null-mutant embryos could be performed to test the specificity of the Pcl antibody.

In case of H3-K27me3, one could have expected to see a reduction but not a complete loss of H3-K27me3 signal because clones of Pcl protein null mutant cells in imaginal discs show reduction but not complete loss of H3-K27me3 (Nekrasov et al. 2007). In parallel, the amounts of the PRC2 components E(z) and Esc have been analyzed and did not seem to be decreased upon Pcl knock-down, as well.

To draw further conclusions from these knock-down experiments, knock-down efficiency need to be improved. Despite the use of different promoters controlling the expression of Gal4 and the use of different Pcl-RNAi strains, no significant decrease has been detected. However, I was able to show that Polycomb target genes are misexpressed in imaginal discs of Pcl knock-down larvae and the degree of misexpression varies upon knock-down efficiency. The abundance of E(z) and Esc components, however, do not seem to vary upon Pcl knock-down. However, as Pcl did not seem to be knocked down in these experiments, no strong conclusions can be made here.

4.2 Knock-out of Pcl in *Drosophila* imaginal discs and effects on PcG protein mediated gene repression

To analyze the phentoype of a *Pcl* null mutant, I analyzed Pcl^{21M22} mutants (Nekrasov et al. 2007). The Pcl^{21M22} allele carries a premature stop codon and embryos homozygous for this mutation lack detectable Pcl protein on western blots (Nekrasov et al. 2007). Pcl^{21M22} homozygous animals can be rescued with a construct expressing N-terminally tagged Pcl, and are healthy and fertile (Nekrasov et al. 2007). As homozygosity of mutant Pcl is lethal in the embryo stage, stocks heterozygous for the mutant Pcl form accompanied by FRT42D recombination sites were crossed with flies bearing the gene for Flp recombinase under a heat-shock promoter on the first chromosome. On the second chromosome, a F42D recombination

site was neighbored to the gene for ubi-GFP. Offspring of these strains bear the gene for the heat-shock inducible recombinase on the first chromosome. After recombination, cell clones lacking endogenous Pcl are marked by the relative levels of GFP expression. Figure 9 shows a scheme of the mitotic recombination of the alleles leading to homozygous cell clones.

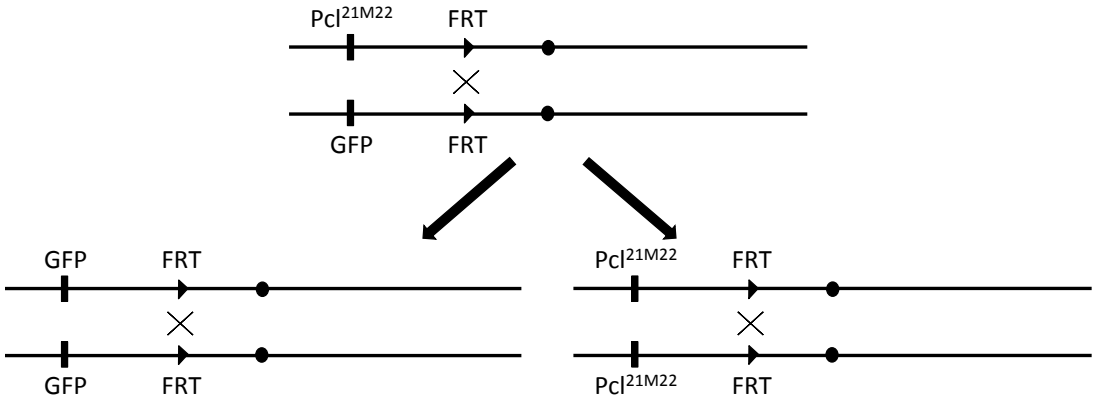

Figure 9: The *GFP* gene (GFP), and the *Pcl* ^{21M22} allele are located on the second chromosome, each on a separate allele (black lines, centromere shown as a circle). Both genes are distal to F42D recombination sites (FRT) to allow heat-shock induced recombination between both alleles, accomplished by the recombinase at the FRT42 recombination sites. After mitotic cell division, recombinant clones are generated with either two wild-type *Pcl* genes or two *Pcl* ^{21M22} genes. Clones homozygous for the wild-type *Pcl* gene are also homozygous for *GFP* and therefore express double the amount of GFP of cells where no recombination took place and where the cells are thus heterozygous for *GFP*. In contrast, clones that are homozygous for *Pcl* ^{21M22} are also lacking *GFP* and do not express GFP protein.

Figure 10 shows imaginal discs of eye, antenna and legs (A) and of wing (B), which were isolated from third instar larvae obtained from the cross described before after heat-shock induced recombination. The discs were stained with antibodies against Ubx, which is a target gene of Polycomb mediated repression. In imaginal discs of third instar larvae, Ubx is repressed as shown in Figure 4 "control". In clones, which are homozygous for the protein negative allele and therefore lack Pcl (marked by the lack of GFP expression), the *Ubx* gene is derepressed. This result shows that Pcl is essential for efficient repression of Polycomb target genes.

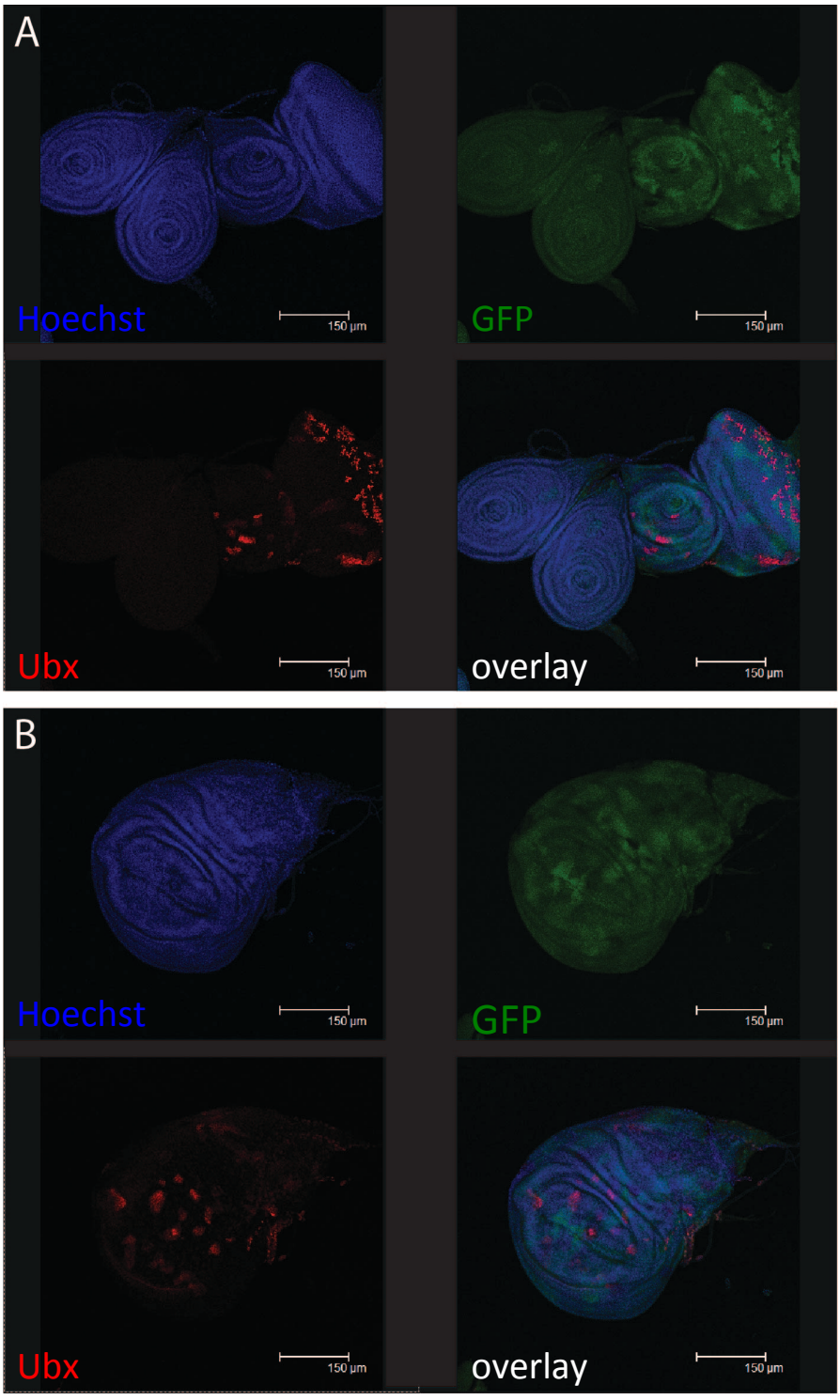

Figure 10: Imaginal discs of third instar larvae obtained from the cross of hs122; F42D ubi-GFP virgins with males heterozygous for *Pcl*^{21M22} neighbored by F42D recombination sites. The larvae were heat-shocked in the first instar larval stage for 1 h at 37°C to induce recombination and imaginal discs of wing, first and second leg, eye and antenna were isolated 72 h after induction of Flp recombinase expression. The discs were fixed in 4% formaldehyde final concentration. Imaginal discs of eye, antenna and legs (A) and wing (B) are shown. DNA was stained with Hoechst (blue) and a-Ubx antibodies were used to detect Ubx expression (red). Cell clones homocygous for the *Pcl*^{21M22} allele are marked by the absence of GFP (green) and do not have the wild type *Pcl* gene. Overlays show the co-localization of the signals. w1118 larvae served as a control. Scale bars represent 150 µm.

4.3 Recruitment of Pcl to PREs

Savla et al. 2008 studied the localization of Pcl to different PREs of the *Ubx* gene, which is known to be a target of PcG protein mediated repression in wing imaginal discs during the development of *Drosophila melanogaster* (Beuchle et al. 2001). He did so to reveal whether Pcl in wing imaginal discs is co-localized with PRC2 complexes and E(z) as a core component of PRC2, as it is assumed that Pcl either is only temporarily associated with PCR2 or is part of only a fraction of PRC2 acting on PREs (Savla et al. 2008).

Nekrasov, on the other hand, focused on alterations of PcG protein recruitment and histone modifications due to Pcl knock-out on a broad selection of PREs of PcG target genes (Nekrasov et al. 2007).

Despite these studies, the localization of Pcl to PcG target genes other than *Ubx* has not yet been analyzed. It can be assumed that Pcl is recruited to PREs of PcG target genes, as it has been shown previously that Pcl is important for efficient Polycomb repression (Nekrasov et al. 2007).

To verify this statement, chromatin immunoprecipitation (ChIP) assays were performed to localize Pcl binding sites.

Chromatin from stocks heterozygous for NTAP-tagged Pcl was prepared from imaginal discs as described in 3.7. By sonication, the chromatin was sheared into fragments ranging from 100 to 2,000 base pairs as shown in Figure 11. The fragment size of chromatin is critical for ChIP experiments. Large DNA fragments lead to a loss of resolution when localizing proteins that bind to DNA. Small DNA fragments, however, might lead to weak signals during quantification of specific DNA target sites.

Recommended fragment sizes are between 0.1 and 1.0 kb (Fan et al. 2008). The obtained chromatin fragments used for ChIP were in this size range.

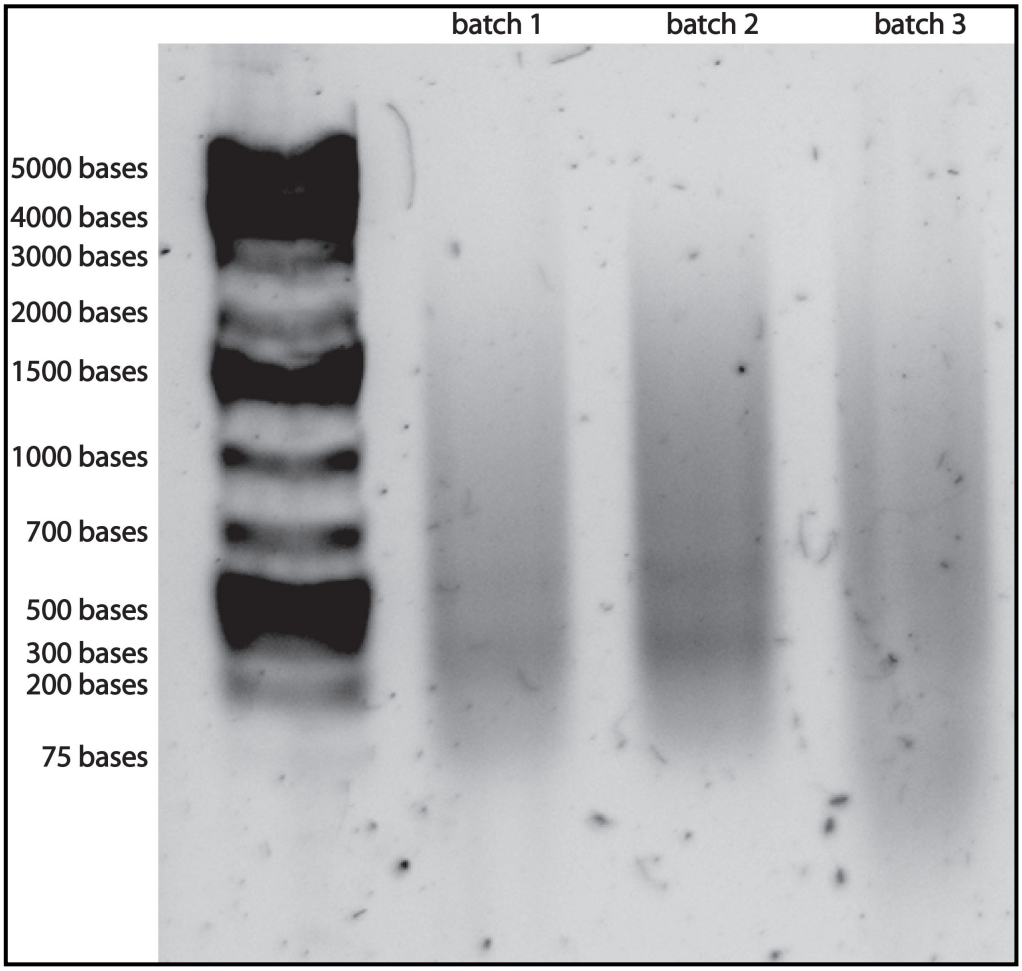

Figure 11: 1% Agarose gel with sonicated chromatin after DNA purification. 3 independent batches were set up after fixation and dissection of discs before sonication. After sonication, chromatin fragments between about 100 to 2000 base pairs were obtained.

Amounts used for ChIP were determined by qPCR in comparison to a sample that had already been tested for ChIP. Optimal results were obtained with 50 µl chromatin from wild-type larvae and 250 µl chromatin from NTAP-Pcl larvae.

After formaldehyde cross-linking the chromatin to temporarily bind NTAP-Pcl, chromatin was immunoprecipitated through the use of IgG beads that bound to the NTAP tagged Pcl. Primers flanking the Polycomb target genes *Ultrabithorax* (*Ubx*), *Abdominal-B* (*Abd-B*), *Sex combs reduced* (*Scr*), *engrailed* (*en*) and *Distal-less* (*Dll*) were used to quantify the amounts of chromatin that was immunoprecipitated. The DNA fragments were quantified via qPCR and adjusted to the total input of chromatin before immunoprecipitation. Chromatin from w^{1118} stocks served as a control.

Figure 12 shows the relative amounts of precipitated chromatin related to the total input before immunoprecipitation. Red columns refers to the NTAP tagged Pcl that was immunoprecipitated

by IgG beads. Blue columns refer to the wild-type Pcl, which should not be immunoprecipitaed and which indicates for unspecific background binding to the beads. The analysed genes are indicated below. The numbers indicate the distance from the transcription start sites in kilobases, which are marked by arrows. Black boxes indicate known PREs.

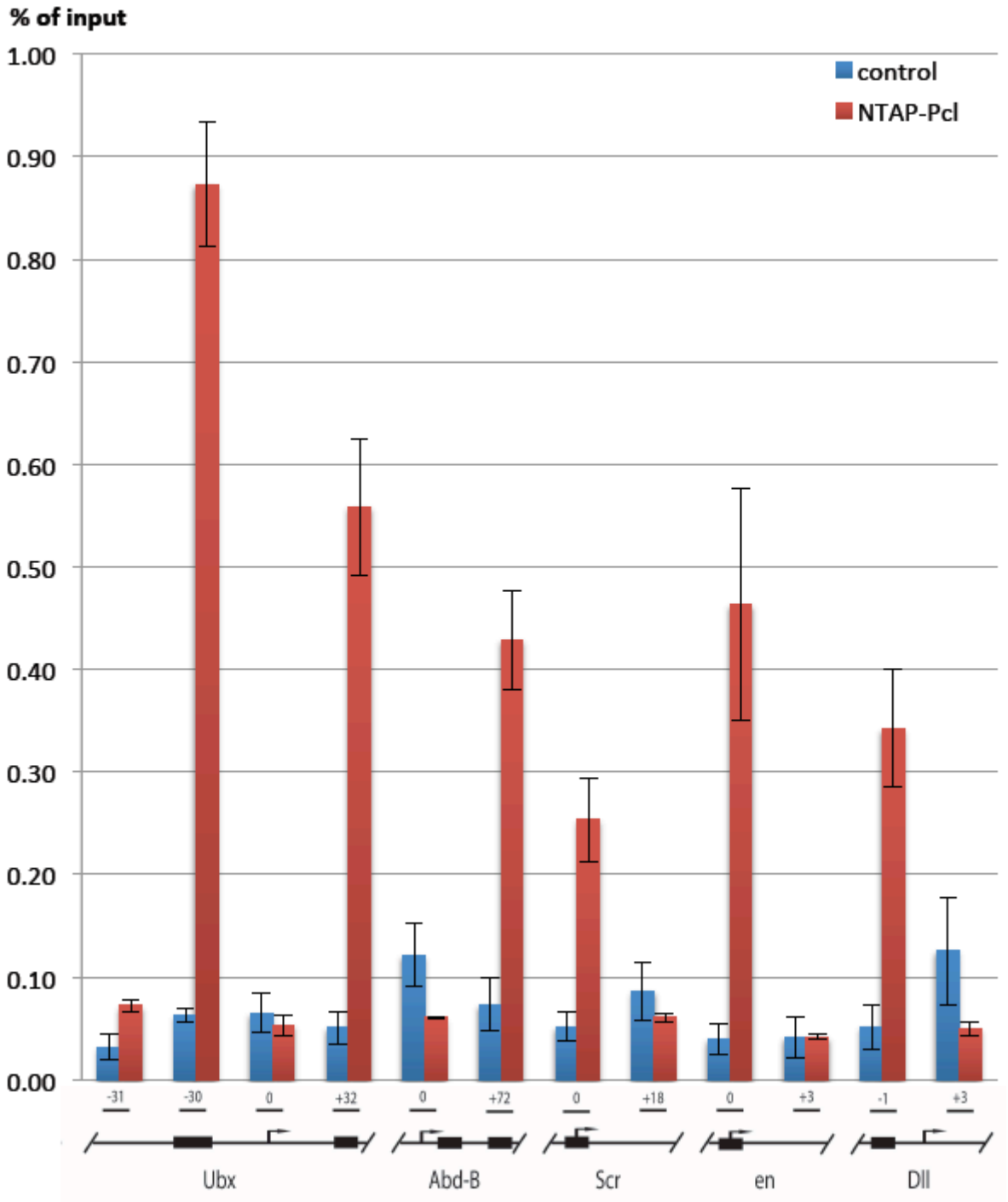

Figure 12: Chromatin isolated after cross-link with NTAP-Pcl and immunoprecipitation with IgG beads. The ordinate indicates the values of immunoprecipitated DNA as a percentage of the input. Chromatin was quantified by qPCR with primers flanking the gene regions described below. Error bars indicate standard deviations from three independent experimental set ups. Black boxes mark the investigated PRE regions, and arrows indicate transcription start sites. The investigated locations are indicated relative to the transcription start site of the corresponding Hox gene in kilobases.

Unfortunately, the values obtained for immunoprecipitated chromatin are not high and less than 1% of the input has been immunoprecipitated. However, detected Pcl after formaldehyde cross-link is up to 17 times higher than in non-PRE regions for *Ubx*, and even up to 27 times higher for *en* (after subtraction of unspecific crosslink). For the other investigated Polycomb target genes, Pcl binding is 3-6 times higher for PREs compared to non-PREs. Therefore, it can be concluded that Pcl predominantly binds to PREs of Hox genes. These results are consistent with previous findings where it has been shown that Pcl is enriched at several *Ubx* PREs (Papp 2006). From Figure 12 it is obvious that this is also true for PREs of *Abd-B*, *Scr*, *en* and *Dll*. However, it is not clear yet how this localization is achieved. One possibility could be the recruitment of Pcl as a component of PRC2. Pho in turn recruits PRC2 to PREs, which is the current opinion of PcG targeting (Anon n.d.). However, it has been shown that the recruitment of Pcl is not affected upon the loss of E(z) (Savla et al. 2008). It is therefore unlikely that Pcl recruitment depends on PRC2. It might also be possible that Pcl bind specifically to PREs like Pho does and helps to recruit PRC2 to PREs. This is the mechanism that has been postulated recently for human Pcl homologues (Casanova et al. 2011). In contrast to this, ChIP experiments on imaginal discs from larvae lacking Pho showed that Pcl is no longer recruited to PREs. Therefore, Pho still might be important for Pcl recruitment to PREs. To address the role of Pcl in the recruitment of PcG proteins to PREs, further experiments need to be done.

4.4 DNA binding properties of Pcl

To assess whether Pcl specifically binds to PREs with its DNA binding regions and not by the recruitment of PRC2, point mutations were introduced into Pcl domains that have been found to be involved in DNA binding (see 1.7).

Amino acids that have been found to be crucial for DNA binding by structural analysis were mutated into alanine. The mutated amino acids were located in the WH domain of Pcl, and mutant Pcl versions were created with mutations in the wing domain or in the helix domain or in both domains at once. Figure 13 shows a proposed model of the solved DNA binding domains of Pcl (purple) in complex with the DNA backbone (orange). The targeted amino acids lysine 650 and arginine 651 are indicated in green for the wing mutant construct (K650 and R651), while arginine 631, glutamine 634 and lysine 637 are indicated in red for the helix mutant construct (R631, Q634 and K637).

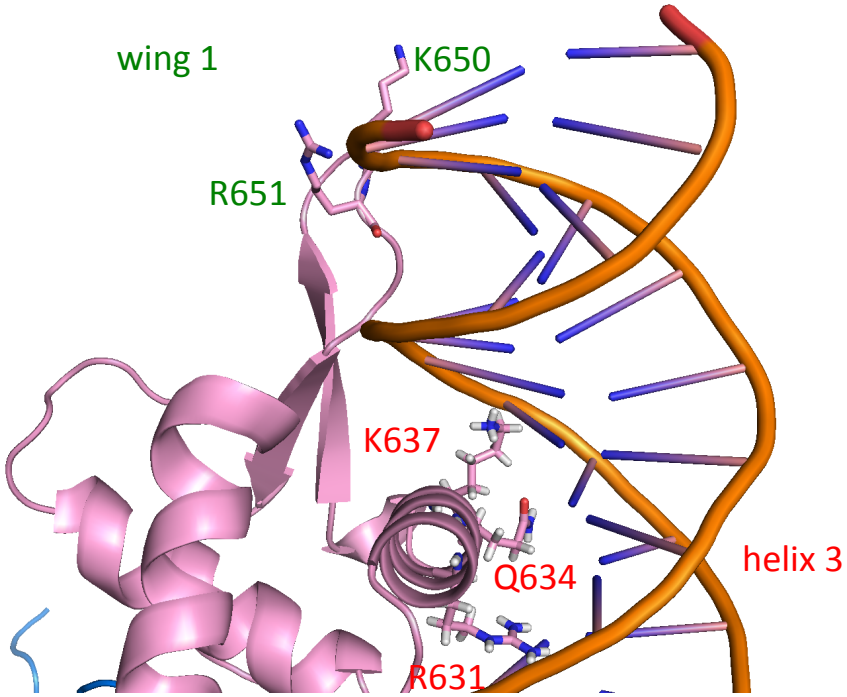

Figure 13: Proposed binding model of the solved Pcl DNA binding structure (purple ribbon model) in complex with DNA. Helix 3 of Pcl, is part of the WH domain, which is the potential DNA binding region. It fits into the major groove of the DNA helix. Wing 1 of Pcl, another part of the WH domain, contacts the DNA minor groove and is likely to be involved in DNA binding as well. For binding studies, amino acids in the wing (labeled in green) and helix domain (labeled in red) has been point mutated into Alanine. Mutated amino acids for the wing mutant construct were lysine 650 (K650) and arginine 651 (R651) and for the helix mutant construct arginine 631 (R631), glutamine 634 (Q634) and lysine 637 (K637).

To analyze the role of this DNA binding activity in Polycomb mediated gene repression, transgenes encoding mutant Pcl versions in the helix or in the wing of the WH domain or in both parts at once were introduced into the fly genome. The mutations were introduced into HA-Pcl-pFBdual as described in 3.14. The coding sequence of the Pcl construct before mutagenesis has been sequenced; it conforms to the sequence L35153 (NCBI GenBank). This sequence bears several silent point mutations throughout the coding sequence and one amino acid exchange in the proline rich domain of Pcl compared to the present sequence (CG5109, http://flybase.org 27/10/2012). Here, the sequence L35153, which is the Pcl sequence used in the experiment, contains an asparagine, whereas the sequence from the flybase databank contains a serine. As this amino acid is not expected to be involved in the DNA binding properties of Pcl and as the used sequence has been shown to be functional, the experiment was continued with this sequence. Nevertheless, we cannot exclude the possibility that silent point mutations can influence protein expression as the sequence can influence mRNA processing or translation due to codon bias.

An additional stop sequence to ensure the termination of Pcl after the coding sequence as well as an additional digestion site to clone the construct into the final pUAST attB vector (Bischof et al. 2007) have also been introduced with this method. Pcl without mutated DNA binding regions served as a wild-type control. An HA-Flag-Flag tag was synthesized as described in 3.12 and ligated into the target vector pUAST attB to tag the wild-type and the mutated Pcl constructs; these were also ligated into the vector as described in 3.15. Accordingly, the mutated and the non-mutated wild-type Pcl constructs were N-terminally tagged with a HA-Flag-Flag tag.

The four constructs – HA-Flag-Flag-Pcl without mutated DNA binding region, with mutations in the wing domain, with mutations in the helix domain and with mutations in both domains – were introduced into the fly genome as described in 3.17. Flies with the Pcl constructs controlled by an UAS were crossed with flies expressing the Gal4 driver under a tubulin or daughterless promoter in order to obtain offspring expressing the Pcl constructs in a wild-type background. This offspring was viable and did not show a Polycomb phenotype, leading to the assumption that the constructs did not harm the animals.

The transgenic flies were crossed over multiple generations to obtain yw hsFlp122 ; F40F42 y^{+} Pcl^{21M22}/F42 ubi-GFP ; UAS-Pcl w+ y+/Gal4 larvae. The crossing steps are described in the supplementary (see 11.6). Unfortunately, the heat-shock induced recombination of the larvae was not performed within this thesis due to a lack of time after the obtention of the required stocks.

5 Summary and outlook

In this thesis, I showed that Pcl is necessary for the repression of Polycomb target genes. This was shown in cell clones lacking Pcl. Furthermore, the misexpression of Pcl target genes in animals with decreased Pcl expression indicated that a distinct amount of Pcl is necessary for efficient Polycomb mediated gene repression.

Furthermore, I showed by ChIP assays that Pcl is specifically recruited to PREs of Polycomb target genes.

To assess whether recently discovered DNA binding regions mediate the binding to PREs, I created several transgenes with point mutations in these regions to decrease DNA binding ability. The transgenes were introduced into the *Drosophila* genome to obtain animals expressing the point mutated Pcl constructs.

Further aims would be to create cell clones in imaginal discs expressing these constructs in a Pcl negative background. This would be done with heat-shock induced recombination of third instar larvae after crossing the stocks that had been performed as described in 3.18.

This experiment could show whether Pcl with decreased DNA binding can rescue cell clones lacking endogenous Pcl. Immunostaining of those discs could be performed to reveal whether these constructs can still mediate their function in gene repression by detecting gene products of Polycomb target genes. If this is the case, it could be concluded that Pcl binding to DNA is not essential for Polycomb mediated gene repression and that *Drosophila* Pcl might not recruit PRC2 to PREs as has been postulated for human Pcl homologues. In contrast, if gene repression of Polycomb target genes fails in cell clones homozygous for the mutated Pcl constructs, the latter assumption would be supported.

Furthermore, ChIP assays could demonstrate whether DNA binding activity is decreased in point-mutated Pcl compared to non-mutated Pcl. Antibodies against the N-terminal HA-Flag-Flag tag of Pcl would be used to immunoprecipitate Pcl and specificity of the antibody could be verified in comparison to parallel experiments with animals expressing non-tagged wild-type Pcl. After immunoprecipitation of mutated and non-mutated N-terminally tagged Pcl, cross-linked DNA could be quantified by qPCR. From these data, it could be concluded whether Pcl specifically binds to PREs by its DNA binding region.

Additionally, ChIP assays could be performed with antibodies against components of PRC2 in order to reveal how the binding of other complex components to PREs is affected by the reduced DNA binding ability of Pcl.

6 Discussion

A common strategy to address the function of a protein is the analysis of cells or organisms lacking this protein followed by an interpretation of the resulting phenotype. This can be achieved through several methods, including recombinase-mediated exclusion of a gene or introduction of a premature stop codon leading to non-translated mRNA.

However, this strategy can be challenging if the gene encodes for an essential protein or for a protein that regulates the early development of the organism, as is often the case for the PcG proteins. As mentioned above, flies homozygously knocked out for Pcl die as embryos, a fact that highlights the importance of Pcl in fly development. Therefore, other alternatives need to be found to obtain organisms that can be analysed. In this thesis, one of these alternatives included knock-out of Pcl in single cell clones in the larval stage of the fly by mitotic recombination.

Another alternative is the knock-down of Pcl by RNA interference. Flies knocked down for Pcl can reach the early pupal stage as mentioned above. Therefore, the effects can be analysed in knock-down larvae. However, elimination of the targeted protein is not complete, which might be critical for proteins working as enzymes. Knock-down efficiency also depends on different variables like the position of the targeted sequence at the gene (Shao et al. 2007). So, for the interpretation of the data, knock-down efficiency needs to be determined and effects of incomplete target degradation needs to be considered when drawing conclusions.

The influence of endogenous protein is also an issue in heterozygously knocked out organisms, as was the case in the DNA binding studies of mutated Pcl constructs by ChIP. Here, chromatin of heterozygous larvae was used due to premature lethality in homozygously knocked out organisms in the case of Pcl. These larvae were also heterozygous for the transgenic N-terminally tagged Pcl, which was precipitated in the experiment. As endogenous Pcl is not tagged, it should not be precipitated and should therefore not be detected in the data. Nevertheless, it cannot be excluded that endogenous Pcl might influence the binding properties by forming multimers with transgenic Pcl or inhibit transgenic Pcl from binding its targets.

In my thesis, I applied different methods. I knocked down Pcl by RNAi interference to reduce the abundance of Pcl in the cell and I eliminated endogenous Pcl by recombination. The methods were chosen according to factors like viability of the animals. However, they also revealed certain issues. For instance, I was able to show that not only a complete elimination but also a decrease of Pcl can impede Polycomb mediated repression. Nevertheless, the western blot analysis did not show a significant decrease of Pcl. This is surprising, as misexpression of

Polycomb target genes has been detected in immunostainings of discs from knock-down larvae, leading to the conclusion that Pcl abundance should be decreased.

This fact highlights the importance of considering different approaches to address an issue in research, which allows to control - and compare - results that have been obtained by using different approaches.

7 Index of tables

8 Index of figures

9 List of abbreviations

Abd-B (Abdominal-B)

ChIP (Chromatin immunoprecipitation)

da-Gal4 (daughterless-Gal4)

Dll (Distal-less)

E(z) (Enhancer of zeste)

en (engrailed)

Esc (Extra sex combs)

H2A-K119ub (ubiquitination at Lysine 119 of Histone H2A)

H3-K9me3 (trimethylation at Lysine 9 of Histone H3)

H4-K20me3 (trimethylation at Lysine 20 of Histone H4)

H3-K27me1 (monomethylation at Lysine 27 of Histone H3)

H3-K27me2 (dimethylation at Lysine 27 of Histone H3)

H3-K27me3 (trimethylation at Lysine 27 of Histone H3)

Hox gene (Homeotic selector gene)

IP (immunoprecipitation)

PcG protein (Polycomb group protein)

Pcl (Polycomblike)

Pcl^{21M22} (protein negative allele with premature stop codon)

PHD (Plant homeo domain)

Pho (Pleiohomeotic)

PhoRC (Pho repressive complex)

PRC1 (Polycomb repressive complex 1)

PRC2 (Polycomb repressive complex 2)

PR-DUB complex (Polycomb repressive deubiquitinase complex)

PRE (Polycomb response elements)

qPCR (quantitative PCR)

RNAi (RNA interference)

Sce/Ring (Sex combs extra; also known as Ring)

Scr (Sex combs reduced)

TrxG protein (Trithorax group protein)

tub-Gal4 (tubulin-Gal4)

UAS (upstream activating sequence)

Ubx (Ultrabithorax)

WH domain (winged helix domain)

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11 Supplementary

11.1 Sequenced open reading frame of HA-Flag-Flag-Pcl

Here, the data after sequencing of N-terminally tagged Pcl without mutated DNA binding regions is shown in 5' to 3' direction. The HA-Flag-Flag tag is marked in blue and the transcription start site is highlighted in bold. The regions that have been mutated are shown in orange for the helix mutation and in red for the wing mutation.

AATTGATGTACCCATACGATGTTCCTGACTATGCGGCTGCAGATTACAAGGATGACGATGACAAGGCA
GCTGATTACAAGGATGACGATGACAAGGCGGCCGCA**ATG**ATGAACAACCATTTTCACTTGCAACACGA
TCATCCRCCGCAGAACGTGGCACATCCGTTCATGCAACAGCCGAGTACCGCTGTccCATCAGCTCCTCCG
GCCACCTACGGCTACTTAGCACAGCCAGCTGGCCAGCAGCCGCAGTGGATGACGACCACCTACCAGATC
CTGCCGCCCAGTGTAGGACCTGCCACGGTGGCCAAGCGATACTATGCCACCACTGGGCCGCAGACCACG
CATCCCACACATCCCAGCACCATCCAGATCACGAACAATTTCGCTCAGCAATCAACGCCACCGAAGCAA
CAGGCGGCAACAAGCTGCAGCCCATTCAAGGCCAACAACATACGAATCATCTCGACGGCGCCAAGTGTA
TACAGTCTGAATAAGCCGCCACAGGAAGCGCACTCCACATATGCTCCCGTTCAGTCGTACTACTTGCCC
AGTGGCGGTGGCCAAACTGCGGGACAAATCAATCTACTGGCGGCCAGCGGAACTGGCAAGCAACTGCA
GCCGCCGCCGCTGGTGCCTGTGACCAATTCCACCTCACCGCCCTCCACCGTCGTGTTAGACCGCATAAAC
ATTTGCATCAATAACCACTATACGGAGACGCCCACTTCGCTAAGTTCATCGCTTACCACGGCCCAACAA
CCTTCGCCCATCATACCGGCCATCCAGCACAAGGCCATCCTGCCCCTCATCGACAGCAGCACGGCGGAT
AGCAGCAGCTGCAGCAGCAGTTCTGTATCCAGCAGCAGTTATAGTGGTACCGCGACCACCTCAGCAGCA
GTTGTGATTGTGGATGAGCCGGATTCTACGACCACAACGCCACAGACACCGCCAACTACGCCGGAGGCC
ATGAGTTCACCCGGTAAAAGTTCGCCCTCACCTCCGCTACTAGCCACGCAATCGCTGCTCAAAGGCGTC
AACTCAATGAAGCCAAGCTTCAAGACCGTCGAAGCAGCACCGCCAACGCCGCCAACACCTCCATCACCA
CCACCACCGCCGCCGGCACCACCAGTTGCCGCTCCCTCGCCAGCGGTCACGTATGCGCTTCAGGAGGACG
TCTTCATCAAATGCAACGATGGCAGATTTTACCTGGGCACCATTATTGACCAGACAAGCGATCAATAT
CTTATCCGCTTTGATGACCAGTCGGAGCAATGGTGTGAGCCGGATAAGCTGCGCAAGCTGGGTGGCGG
TAGCTCGATTACCGCTGGCGGAGGCGGTGCCTCTACCACGGAATCCACGAATACCTCCCCCAGTGGACC
CATGTGCGTGGCCTGCAAGCGATCGGATATCGAAGACGTGGTCGAAATTTGCGAGCGCTGCGGACGTG
GCTATCATCGTGGTTGCACCGTTGAAATTGTTACTGGCAGCGGAATTTGGAGTTGCAAACGCTGCGCT
AAACCCATGAAAATGCAACAGCCTGTCAGCCATAAGATCACAAAACCAGCGGGAATTTGTCGCCAGTT
GCCCTACCATGCGGATAAGCTCAGCTGGGATGAGAAGCATCGGGTCAACGAAGAGCAGATCTACTGCT
ACTGCGGCAAACCGGGAAAATTCGATCACAATATGCTGCAGTGCTGCAAATGTCGGAACTGGTTCCAC

ACCCAATGCATGCAGAACTTCAAGAAGAAGCTGCTGCGCGGCGATATGTTCTTTGTTTTCTGCTGCACG
GTCTGCAACAATGGCATAGAATTCGTTCGTCGTATGCAAATCGAATGGGTGGACGTGCTCCACATTGC
ACTATACAATCTGCGAAAGCATCAGCACCAGAAGTACCATCACCTGCTAAATGACATTTGGCCCTTCAT
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GCGTCTCAAGCAAACCCTTAAGGATTACTCTGACAGATTTGTTTGCGGCAGGGAGTTTAAAAGGGCTC
CGGCCTTCTATGCACTGAGACACAGTGGCCCGCCACACATTCCCAAGGTATTCCTTGAGCCCCACGAGG
AGCTGTCTGATGAGCTGCTCGAAAAAAGATTTAAACTAATGCTCATGCCCGAGGAGCCTGATGAAGGT
GCCAACGAGCTTCCGAAAAGAGTTCCCAAAGATGTGTATGAGTTTAATACAGATGAAGATGACCCAGT
CGAGACCAGCGAGGACGAAATACCCATCAAACAGATTATTGAGAAGGCCAAAAAGCAGGCCGCTCAGA
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GATTGTCCAAACGCTATGACAACAGCCGCAATCACTGTGATCTATCATCCGACGAGAATTCAAGCAGC
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TTGCGCCAGCGACCACAGAAGCAGTCACCCAGCCAAAGCCGCCGGAACTCCACCAGCTCGACGGCAACC
AGCAGCAGCAGCAACGGCATTGGAGCCCCAGGACATTCCATGCTGGACTTGAAGCAGTCGGTGAACAA
GTACTTTGGTGGCGCTATGAATCGCATAGATGCTGGTGAGCCCTTTGCCATTCGGGCCAAGCGGCGCAT
GGGAAATGGACAGGTCCAGTACCTGGTGGAGTGGGGCGGAGATACAGCGACAACGGCGATTGGATTGC
TTGGAAAT

11.2 Oligonucleotides for mutagenesis

Oligonucleotides were ordered from Sigma Aldrich; all sequences are shown in 5' to 3' direction; mismatches are indicated in lower-case.

Introduction of additional stop sequence followed by a XbaI digestion site at the 3'end of Pcl

Pcl STOP XbaI fw	GCG ATT GGA TTG CTT GGA AAT TGA tga tcT aGa CAT ACC GAC TCA TTA TGA ATT TTA TAT TCT TC
Pcl STOP XbaI rv	GAA GAA TAT AAA ATT CAT AAT GAG TCG GTA TGt CtA gat caT CAA TTT CCA AGC AAT CCA ATC GC

Introduction of point mutations into DNA binding regions of Pcl

K650A, R651A fw	GTT TGC GGC AGG GAG TTT gcA gcG GCT CCG GCC TTC TAT GC
K650A, R651A rv	GCA TAG AAG GCC GGA GCC gcT gcA AAC TCC CTG CCG CAA AC
R631A, Q634A, K637A fw	CGA AAC GGC ACT GAT GGA Ggc TCT CAA Ggc AAC CCT Tgc GGA TTA CTC TGA CAG ATT TG
R631A, Q634A, K637A rv	CAA ATC TGT CAG AGT AAT CCg cAA GGG TTg cCT TGA Gag cCT CCA TCA GTG CCG TTT CG

HA-Flag-Flag overlap oligos

HA-2xFLAG f1L	AAT TGA TGT ACC CAT ACG ATG TTC CTG ACT ATG
HA-2xFLAG f2	CGG CTG CAG ATT ACA AGG ATG ACG ATG ACA AG
HA-2xFLAG f3L	GCA GCT GAT TAC AAG GAT GAC GAT GAC AAG GC
HA-2xFLAG r1	CAG CCG CAT AGT CAG GAA CAT CGT ATG GGT ACA TC
HA-2xFLAG r2	AGC TGC CTT GTC ATC GTC ATC CTT GTA ATC TG
HA-2xFLAG r3L	GGC CGC CTT GTC ATC GTC ATC CTT GTA ATC

11.3 Oligonucleotides for sequencing

Oligonucleotides were ordered from Sigma Aldrich; all sequences are shown in 5' to 3' direction.

Sequencing of Pcl forward primers

c.48-68	CGTGGCACATCCGTTCATG
c.528-546	GGTGCCTGTGACCAATTCC
c.1047-1065	CACGTATGCGCTTCAGGAG
c.1502-1520	GCTGGGATGAGAAGCATCG
c.2019-2038	CCACGAGGAGCTGTCTGATG
c.2534-2554	CGCAGGGACGAAGTATTTCTG
c.3007-3025	CGCATAGATGCTGGTGAGC

Sequencing of Pcl reverse primers

c.131-114	GCTGGCTGTGCTAAGTAG
c.759-741	GCTGCTGGATACAGAACTG
c.1138-1120	GATATTGATCGCTTGTCTG
c.1725-1708	GATTTGCATACGACGAAC
c.2186-2169	GGTATTTCGTCCTCGCTG
c.2738-2719	ACCTCAATGGCCGTGGTCAG

Sequencing of pFBdual

pFB fw, 4433-4454	GTGTTGGGTTGAATTAAAGGTC
pFB rv, 4752-4731	CTCTACAAATGTGGTATGGCTG

Sequencing of pUAST attB

pUAST attB fw	AACTACTGAAATCTGCC
pUAST attB rv	TGTAGGTAGTTTGTCC

11.4 Oligonucleotides for qPCR

Oligonucleotides were ordered from Sigma Aldrich; all sequences are shown in 5' to 3' direction.

Ubx -31 fw	GCAGCATAAAACCGAAAGGA
Ubx -31 rv	GATAGGAGACTTACAAACCGC
Ubx -30 fw	TAGTCTTATCTGTATCTCGCTCTTA
Ubx -30 rv	CTCAATAGCCGTGAAACCAAGAC
Ubx 0 fw	TCCAATCCGTTGCCATCGAACGAAT
Ubx 0 rv	TGAGTTGAGTGAGCTGAGCCGGAATT
Ubx +30 fw	CCATAAGAAATGCCACTTTGC
Ubx +30 rv	TAGTGTCACTCTCTCACTCTC
Abd-B 0 fw	CACTTTCGAGCAAGAGCG
Abd-B 0 rv	CGACAGACACTTAGCGACAA

Abd-B +72 fw	GGAATACCGCACTGTCGTAGG
Abd-B +72 rv	AAGTGAGGTACTACCGACG
Scr 0 fw	GAAGTGCGCCACGTTCAAT
Scr 0 rv	TTGCTCACGCTCTCTCTCCT
Scr +18 fw	CCTGAGTGTCCAATCAACAGG
Scr +18 rv	TGACACGATGCGGCGATGTA
en 0 fw	GTTCACTCCCTCTGCGAGTAG
en 0 rv	CTGCAAAGTTAGACGCAAAAG
en +3 fw	CGCCTTAAGGTGAGATTCAGTT
en +3 rv	TGGTTTTATAACTGTGGCGG
Dll -1 fw	CCTAGCCACAAAGCGACATT
Dll -1 rv	TCAAAGACGAGAGTCGTCCC
Dll +3 fw	ATCCGGAGCACCTATCAGC
Dll +3 rv	TCTTGAGGATGAGGCCGATGG

11.5 Overview of crosses for Pcl Knock-down

Males from 13 different stocks bearing the Pcl-RNAi transgene were crossed with virgins expressing Gal4 under the control of the tubulin or daughterless promotor in order to screen for offspring with efficiently knocked down Pcl. To drive Pcl-RNAi expression, virgins homozygous for da-Gal4 or virgins expressing tub-Gal4 over the TM6B balancer were used. This balancer is marked by a dominant gene called *tubby*, which leads to flies with shorter abdomen ("tubby" flies). Only the non-"tubby" offspring express the tub-Gal4 driver and are knocked down for Pcl. In contrast, the "tubby" offspring, should not express the Pcl-RNAi transgene. The TM6B balancer was also marked by GFP.

To see whether strains bearing the UAS-Pcl transgene are homozygous for this allele, crosses were performed with flies of the strain w^{1118}. This strain has a mutation in the *white* gene, which is responsible for the eye pigmentation of the fly, and therefore has white eyes. The UAS-Pcl transgene is marked by $white^{+}$ that turns their eyes red in a $white^{-}$ background. Flies without the transgene therefore have white eyes. As a result, when flies that are homozygous for the transgene were crossed with $white^{-}$ flies, all of the offspring should have at least one $white^{+}$ allele and should therefore have reddish eyes. In contrast, when flies that are heterozygous for the transgene were crossed with *white*- flies, some of the offspring will not have the $white^{+}$ allele and should therefore have white eyes. According to this experiment, stocks that are homozygous for the transgene were identified and indicated as "homozygous". The remaining stocks, which were not indicated to be homozygous, were identified as heterozygous according to this experiment. The performed knock-down crosses and a short description of the offspring obtained after crossing with the daughterless Gal4 driver or the tubulin Gal4 driver are shown in Table 1. The UAS-Pcl-RNAi stocks N°1-N°8 are described in Savla et al. 2008; the UAS-Pcl-RNAi stocks N°9-N°11 and N°12-N°13 were ordered from Bloomington *Drosophila* Stock Center, Indiana University (http://flystocks.bio.indiana.edu), and from Vienna *Drosophila* RNAi Center (http://stockcenter.vdrc.at), respectively. These stocks are described under the respective stock number of the corresponding order service, which is indicated in Table 1.

Table 1: Table 1: Overview of the accomplished crosses to obtain larvae, which are knocked down for Pcl. For the knock-down, the UAS-Gal4 system has been used (Duffy 2002). The UAS-Pcl-RNAi stocks N°1-N°8 are a gift from Richard Jones' research group and are described in (Savla et al. 2008), the UAS-Pcl-RNAi stocks N°9-N°11 are described at Bloomington *Drosophila* Stock Center under 31189, 33945 and 33946, the UAS-Pcl-RNAi stocks N°12-N°13 are described at Vienna *Drosophila* RNAi Center under v22004 and v22005. The driver Gal4 is controlled by a daughterless or by a tubulin promoter. Phenotypes of the obtained offspring have been described to estimate the efficiency of Pcl knock-down. According to these observations, the knock-down larvae from the crosses marked in bold were selected for further investigation because those bore the strongest phenotype.

Pcl-RNAi stock N°	**Description of UAS-Pcl-RNAi stock**	$\frac{w}{w}; \frac{Sp}{CyO}; \frac{daGal4\ (w+)}{daGal4\ (w+)}$	$\frac{w}{w}; \frac{+}{+}; \frac{tubGal4\ (w+)}{TM6B\ GFP\ (w+)}$
1	pWIZ-Pcl on chromosome III (homozygous)	All flies hatched and had no Polycomb phenotype.	All flies hatched and had no Polycomb phenotype.
2	pWIZ-Pcl on X chromosome	Males hatched and were not affected by RNAi mediated knock-down of Pcl as they did not receive the X chromosome with the Pcl-RNAi transgene. Female pupae developed into the late developmental stage.	No cross performed.
3	pWIZ-Pcl on chromosome III (homozygous)	No flies hatched but the pupae developed into the late pupal stage. About half of the male pupae had extra sex combs on the second leg.	No cross performed.

Pcl-RNAi stock N°	**Description of UAS-Pcl-RNAi stock**	**$\frac{w}{w}; \frac{Sp}{CyO}; \frac{daGal4\ (w+)}{daGal4\ (w+)}$**	**$\frac{w}{w}; \frac{+}{+}; \frac{tubGal4\ (w+)}{TM6B\ GFP\ (w+)}$**
4	pWIZ-Pcl on chromosome II	No flies hatched* and most of the pupae died in the early pupal stage. Almost all male pupae that developed into the late pupal stage had extra sex combs on the second leg.	"Tubby" but also non-"tubby" flies hatched. Half of the hatched flies had curled wings, which indicates that those flies obtained a second chromosome balancer named CyO*. Only hatched flies were "tubby", a fact confirmed by GFP expression. These flies did not show a Polycomb phenotype. Flies that died in the early pupal stage did not express GFP and were not "tubby". It is therefore assumed that those pupae expressed the Gal4 driver.
5	**pWIZ-Pcl on chromosome II (homozygous)**	**No flies hatched and most of the pupae died in the early pupal stage. All male pupae that developed into the late pupal stage had extra sex combs on the second leg.**	**Only "tubby" flies hatched, and these flies expressed GFP. Flies that died in the early pupal stage did not express GFP and were not "tubby". It is therefore assumed that those pupae expressed the Gal4 driver.**

Pcl-RNAi stock N°	Description of UAS-Pcl-RNAi stock	$\frac{w}{w};\frac{Sp}{CyO};\frac{daGal4\ (w+)}{daGal4\ (w+)}$	$\frac{w}{w};\frac{+}{+};\frac{tubGal4\ (w+)}{TM6B\ GFP\ (w+)}$
6	**pWIZ-Pcl on chromosome II (homozygous)**	**No flies hatched and most of the pupae died in the early pupal stage. Most of the male pupae that developed into the late pupal stage had extra sex combs on the second leg.**	**Only "tubby" flies hatched, and these flies expressed GFP. Flies that died in the early pupal stage did not express GFP and were not "tubby". It is therefore assumed that those pupae expressed the Gal4 driver.**
7	pWIZ-Pcl on chromosome II (homozygous)	No flies hatched and most of the pupae died in the early pupal stage. About two thirds of the male pupae that developed into the late pupal stage had extra sex combs on the second leg.	No cross performed.
8	pWIZ-Pcl on X chromosome	Males hatched and were not affected by RNAi mediated knock-down of Pcl as they did not receive the X chromosome with the Pcl-RNAi transgene. Female pupae developed into the late developmental stage.	No cross performed.
9	Bloomington 31189	All flies hatched and had no Polycomb phenotype.	All flies hatched and had no Polycomb phenotype.

Pcl-RNAi stock N°	Description of UAS-Pcl-RNAi stock	$\frac{w}{w};\frac{Sp}{CyO};\frac{daGal4\ (w+)}{daGal4\ (w+)}$	$\frac{w}{w};\frac{+}{+};\frac{tubGal4\ (w+)}{TM6B\ GFP\ (w+)}$
10	Bloomington 33945	No flies hatched but the pupae developed into the late pupal stage. All male pupae had extra sex combs on the second leg.	Only "tubby" flies hatched, and these flies expressed GFP. Flies that died in the late pupal stage did not express GFP and were not "tubby" but had extra sex combs on the second legs. It is therefore assumed that those pupae expressed the Gal4 driver.
11	Bloomington 33946	Only few pupae developed into the late pupal stage and most of the pupae died in the early pupal stage. Male pupae had extra sex combs on the second leg. Two males hatched and one had extra sex combs on the second and third legs; the other one did not have extra sex combs.	Almost all flies hatched as only a few of them had the Pcl-RNAi transgene AND the tub-Gal4 driver because both of the paternal stocks were heterozygous. Offspring with the Pcl-RNAi transgene and the driver died in the late pupal stage and had extra sex combs on the second legs.

Pcl-RNAi stock N°	Description of UAS-Pcl-RNAi stock	$\frac{w}{w}; \frac{Sp}{CyO}; \frac{daGal4\ (w+)}{daGal4\ (w+)}$	$\frac{w}{w}; \frac{+}{+}; \frac{tubGal4\ (w+)}{TM6B\ GFP\ (w+)}$
12	VDRC v22004	No flies hatched and all pupae died in the early pupal stage.	Only "tubby" flies hatched, and these flies expressed GFP. Flies that died in the early pupal stage did not express GFP and were not "tubby". It is therefore assumed that those pupae expressed the Gal4 driver.
13	VDRC v22005	No flies hatched and all pupae died in the early pupal stage.	Only "tubby" flies hatched, and these flies expressed GFP. Flies that died in the early pupal stage did not express GFP and were not "tubby". It is therefore assumed that those pupae expressed the Gal4 driver.

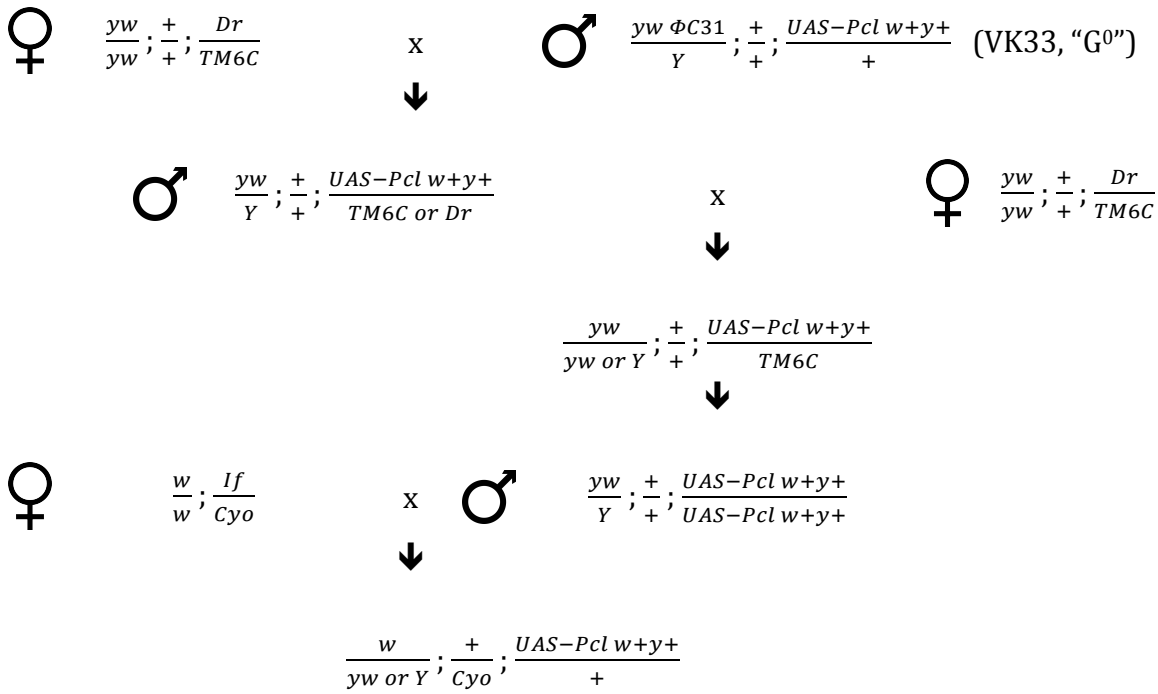
*These results are conflicting because it is obvious from the crossing experiments with w^{1118} virgins that the Pcl-RNAi strain is heterozygous. This was also confirmed by the obtention of flies with curled wings, which shows that the Pcl-RNAi transgene was probably over the second chromosome balancer CyO. The fact that no flies hatched indicates that all of the offspring have probably received the UAS-Pcl-RNAi transgene and none of them the CyO balancer. As this seems to be unlikely if all flies were heterozygous for the UAS-Pcl-RNAi transgene, it is probable that some of the UAS-Pcl-RNAi transgenic flies lost the balancer and turned homozygous.

11.6 Crossing schemes

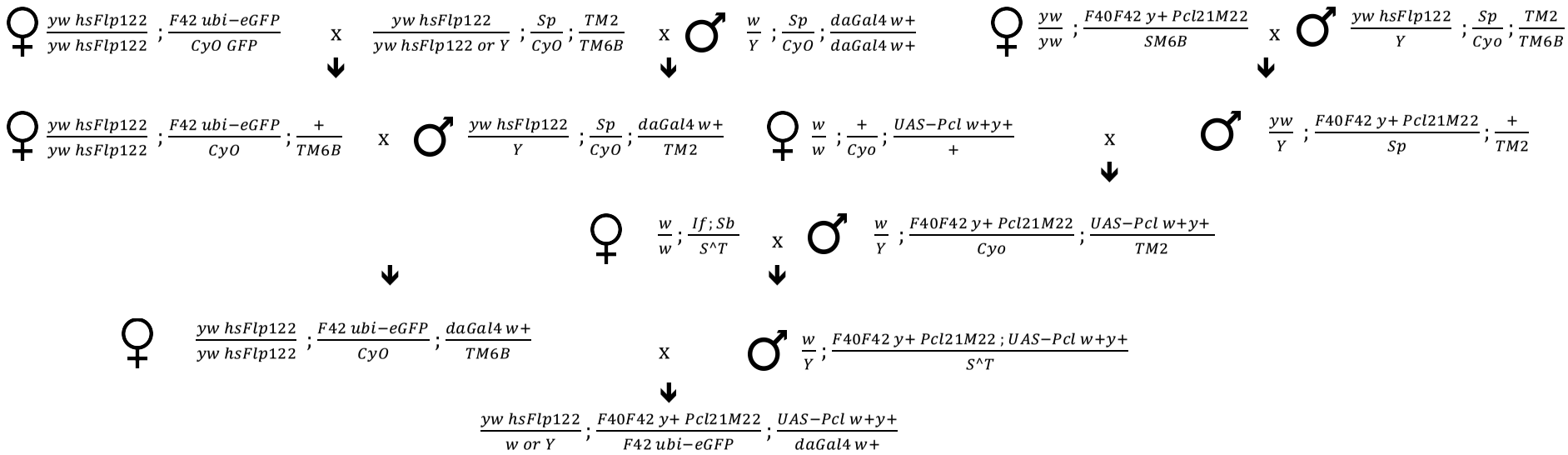
Below are the crossing schemes used to obtain larvae to create cell clones homozygous for mutated Pcl. Cell clones are created by a heat-shock induced recombinase.

Scheme A shows the crosses of injected VK33 males with yw ; Dr /TM6C virgins to screen for transgenic flies after plasmid injection. Only males of the transgenic offspring were selected to cross out the ΦC31 integrase to avoid unwanted recombination. These were crossed again with yw ; Dr/TM6C virgins and the offspring was kept as a stock. Males of this stock were then crossed with w; If/Cyo to introduce the Cyo balancer. In the crossing schemes B and C, a Gal4 driver to induce Pcl expression was introduced. To enable heat-shock induced recombination, the heat-shock induced Flp122 as well as F42 recombination sites on both second chromosomes were introduced. These recombination sites were either neighboured by *Pcl*^{21M22} or a *GFP* gene to identify cell clones after recombination. Different promoters to adjust Gal4 expression and therefore Pcl expression controlled the Gal4 drivers: Scheme B shows the crosses to introduce the da-Gal4 driver and Scheme C the crosses for the tub-Gal4 driver.

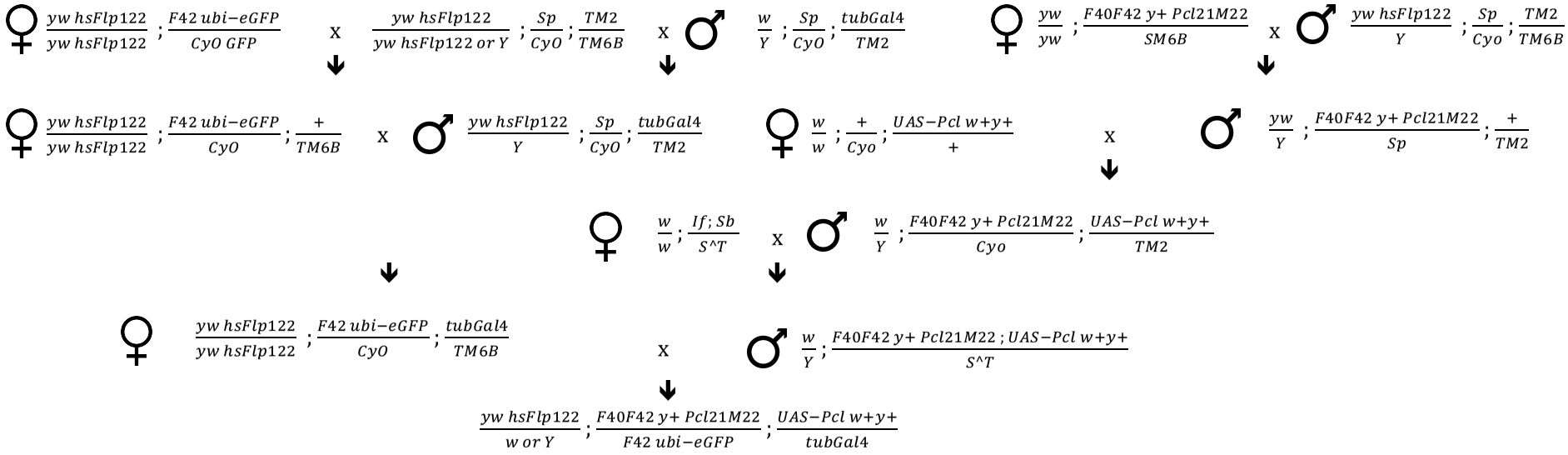
A



B


♀ yw hsFlp122 / yw hsFlp122 ; F42 ubi−eGFP / CyO GFP x yw hsFlp122 / yw hsFlp122 or Y ; Sp / CyO ; TM2 / TM6B x ♂ w / Y ; Sp / CyO ; daGal4 w+ / daGal4 w+
♀ yw / yw ; F40F42 y+ Pcl21M22 / SM6B x ♂ yw hsFlp122 / Y ; Sp / Cyo ; TM2 / TM6B
♀ yw hsFlp122 / yw hsFlp122 ; F42 ubi−eGFP / CyO ; + / TM6B x ♂ yw hsFlp122 / Y ; Sp / CyO ; daGal4 w+ / TM2
♀ w / w ; + / Cyo ; UAS−Pcl w+y+ / + x ♂ yw / Y ; F40F42 y+ Pcl21M22 / Sp ; + / TM2
♀ w / w ; If ; Sb / S^T x ♂ w / Y ; F40F42 y+ Pcl21M22 / Cyo ; UAS−Pcl w+y+ / TM2
♀ yw hsFlp122 / yw hsFlp122 ; F42 ubi−eGFP / CyO ; daGal4 w+ / TM6B x ♂ w / Y ; F40F42 y+ Pcl21M22 ; UAS−Pcl w+y+ / S^T
yw hsFlp122 / w or Y ; F40F42 y+ Pcl21M22 / F42 ubi−eGFP ; UAS−Pcl w+y+ / daGal4 w+

C


♀ yw hsFlp122 / yw hsFlp122 ; F42 ubi−eGFP / CyO GFP x yw hsFlp122 / yw hsFlp122 or Y ; Sp / CyO ; TM2 / TM6B x ♂ w / Y ; Sp / CyO ; tubGal4 / TM2
♀ yw / yw ; F40F42 y+ Pcl21M22 / SM6B x ♂ yw hsFlp122 / Y ; Sp / Cyo ; TM2 / TM6B
♀ yw hsFlp122 / yw hsFlp122 ; F42 ubi−eGFP / CyO ; + / TM6B x ♂ yw hsFlp122 / Y ; Sp / CyO ; tubGal4 / TM2
♀ w / w ; + / Cyo ; UAS−Pcl w+y+ / + x ♂ yw / Y ; F40F42 y+ Pcl21M22 / Sp ; + / TM2
♀ w / w ; If ; Sb / S^T x ♂ w / Y ; F40F42 y+ Pcl21M22 / Cyo ; UAS−Pcl w+y+ / TM2
♀ yw hsFlp122 / yw hsFlp122 ; F42 ubi−eGFP / CyO ; tubGal4 / TM6B x ♂ w / Y ; F40F42 y+ Pcl21M22 ; UAS−Pcl w+y+ / S^T
yw hsFlp122 / w or Y ; F40F42 y+ Pcl21M22 / F42 ubi−eGFP ; UAS−Pcl w+y+ / tubGal4