Application of RNA interference for the study of lethal genes and dynamic processes

Dissertation
zur Erlangung des mathematisch-naturwissenschaftlichen Doktorgrades
“Doctor rerum naturalium”
der Georg-August-Universität Göttingen

im Promotionsprogramm Biologie
der Georg-August University School of Science (GAUSS)

vorgelegt von
Julia Ulrich

aus Kiew, Ukraine
Göttingen, 2015

Betreuungsausschuss

Prof. Dr. Gregor Bucher

Abt. Entwicklungsbiologie, Johann-Friedrich-Blumenbach-Institut für Zoologie und Anthropologie, Georg-August-Universität Göttingen

Prof. Dr. Ernst A. Wimmer

Abt. Entwicklungsbiologie, Johann-Friedrich-Blumenbach-Institut für Zoologie und Anthropologie, Georg-August-Universität Göttingen

Prof. Dr. Andreas Vilcinskas

Abt. Angewandte Entomologie, Projektgruppe “Bio-Ressourcen”, Fraunhofer Institut für Molekularbiologie und Angewandte Ökologie, Universität Gießen

Mitglieder der Prüfungskommission

Referent/in: Prof. Dr. Gregor Bucher
Abt. Entwicklungsbiologie, Johann-Friedrich-Blumenbach-Institut für Zoologie und Anthropologie, Georg-August-Universität Göttingen

Korreferent/in: Prof. Dr. Ernst A. Wimmer
Abt. Entwicklungsbiologie, Johann-Friedrich-Blumenbach-Institut für Zoologie und Anthropologie, Georg-August-Universität Göttingen

Weitere Mitglieder der Prüfungskommission

Prof. Dr. Reinhard Schuh, Abt. Molekulare Entwicklungsbiologie, Max-Planck-Institut für biophysikalische Chemie

Dr. Roland Dosch, Institut für Entwicklungsbiochemie, Universitätsmedizin Göttingen

PD Dr. Gerd Vorbrüggen, Abt. Molekulare Zelldynamik, Max Planck Institut für Biophysikalische Chemie

Prof. Dr. Daniel J. Jackson, Abt. Geobiologie-Courant Forschungszentrum Geobiologie, Georg-August-Universität Göttingen

Tag der mündlichen Prüfung: 20.07.2015

Herewith I declare, that I prepared the Dissertation

"Application of RNA interference for the study of lethal genes and dynamic processes"

on my own and with no other sources and aids than quoted.

______________________ Göttingen, June 4th, 2015

Julia Ulrich

Für

meine Familie

Danksagung

An erster Stelle möchte ich Herrn Prof. Dr. Gregor Bucher für die ausgezeichnete Betreuung meiner Arbeit danken. Ich danke ihm für die wissenschaftlichen Hilfestellungen, das entgegengebrachte Vertrauen an diesem Projekt selbständig arbeiten zu dürfen und die immer vorhandene Unterstützung, wann immer sie nötig war. Desweiteren danke ich ihm für die Möglichkeit meine Ergebnisse bei nationalen und internationalen Tagungen präsentieren zu dürfen.

Danken möchte ich auch Prof. Dr. Andreas Vilcinskas für kritische und weiterführende Anmerkungen zu meiner Arbeit sowie die Möglichkeit an diesem Projekt arbeiten zu dürfen.

Ich bedanke mich bei Herrn Prof. Dr. Ernst A. Wimmer für die anregenden Diskussionen und konstruktive Anmerkungen zu dieser Arbeit während der Labor-Seminare und im Rahmen der Thesis Committee Meetings.

Herzlichen Dank den Mitarbeitern und Mitarbeiterinnen der gesamten Abteilung Entwicklungsbiologie für die äußerst schöne Arbeitsatmosphäre, die stete Hilfsbereitschaft und Unterstützung. Mein ganz besonderer Dank geht dabei an das gesamte Labor 3: Peter Kitzmann, Janna Siemanowski, Georg Oberhofer, Yong Gang, Salim Ansari, Claudia Hinners und Jürgen Dönitz. Ohne euch wäre die Zeit im Labor nur halb so schön gewesen!

Elke Küster danke ich für das Ansetzen und Pflegen meiner vielen Käferkreuzungen und Claudia Hinners für das Herstellen von zahlreichen Doppelstrang RNAs.

Ein besonderer Dank gilt auch Jürgen Dönitz für die kritische Durchsicht dieser Arbeit und sehr konstruktive Anmerkungen.

Ganz besonders möchte ich meinen Eltern und meiner Schwester danken, die mir dieses Studium erst ermöglicht haben und mich immer unterstützt und an mich geglaubt haben.

Zum Abschluss möchte ich mich bei meinem Freund bedanken. Danke, dass du mich immer ermutigt, an mich geglaubt und mich in schweren Zeiten immer zum Lachen gebracht hast!

Table of contents

1 Summary 5
2 Introduction 7
2.1 RNAi based strategies of plant protection 7
2.1.1 RNA interference 8
2.1.2 Application of RNAi in pest control and current limitations 10
2.1.2.1 RNAi upon dsRNA ingestion 11
2.1.2.2 Off targets 11
2.1.2.3 Endogenous plant RNAi mechanism 12
2.1.2.4 Resistance development 12
2.1.2.5 Target gene selection 12
2.1.3 *Tribolium* as model system and screening platform 13
2.1.4 Potential RNAi target genes 14
2.2 Aims 15
2.2.1 Identification of novel RNAi target genes for pest control 15
2.3 Temporal and/or spatial regulation of RNAi mechanism 17
2.3.1 Suppression of the RNAi pathway 19
2.3.1.1 FHV B2 20
2.3.1.2 DCV1A 20
2.3.1.3 CrPV1A 21
2.3.1.4 Nora virus VP1 21
2.3.1.5 TCV p38 22
2.3.1.6 PVX p25 22
2.4 Aims 23
2.4.1 Establishment of a tool for temporal and/or spatial regulation of RNAi 23
3 Materials and Methods 25
3.1 Strains 25
3.2 Molecular cloning 26
3.3 RNA interference 26
3.4 GO term clustering and identification of novel potential RNAi target genes 28
3.5 Off target analysis 28
3.6 Phylogenetic analysis 29

3.7 Transgenesis 29
3.7.1 Constructs 29
3.7.2 Germline transformation and transgenic lines 31
3.7.3 Transformation markers and GFP analysis 32
3.7.4 *Tribolium* genetic crosses 32
3.7.5 Heat shock conditions 33
4 Results 34
4.1 Large scale RNAi screen identifies novel RNAi target genes 34
4.1.1 Double RNAi led to additive but not to synergistic effects 37
4.1.2 Degree of sequence conservation does not strongly influence the number of off targets 38
4.1.3 GO term clusters identify the proteasome as prime insecticide target 41
4.2 Identification of an efficient RNAi suppressor protein in *Tribolium castaneum* 45
4.2.1 VSRs did not suppress silencing of heat shock activated EGFP 45
4.2.2 CrPV1A efficiently suppressed the knockdown of UAS-Gal4 expressed tGFP 48
4.2.3 Confirmation of CrPV1A as an efficient inhibitor 51
4.2.4 Silencing of endogenous *Dicer-2* gene failed to suppress *Tc-ebony* RNAi 52
4.2.5 CrPV1A does not interfere with development and reproduction 54
4.3 Temporal restriction of RNAi 57
4.3.1 Starting of an RNAi pulse by injection in staged embryos failed 57
4.3.2 Heat shock induced VSR expression rescues abdominal segmentation of *Tc-prd* RNAi embryos 60
5 Discussion 64
5.1 RNAi target genes for pest control 64
5.1.1 Necessity for more efficient target genes 64
5.1.2 The large scale RNAi screen reveals novel genes for application in pest control 65
5.1.2.1 Tests in further pest species are required 65
5.1.2.2 Off target effects on non-target organisms cannot be excluded but are improbable 66
5.1.3 GO term clusters are predictive for efficient RNAi target genes and identify the proteasome as prime target 67

5.1.3.1 Potential reasons for the failure to identify predictive GO term clusters for the top eleven RNAi target genes 69
5.1.4 Possibilities to increase the efficacy of the identified RNAi target genes 69
5.1.5 The identification of more efficient RNAi target genes is unlikely...........70
5.2 Investigation of RNAi suppressors in *Tribolium*...71
5.2.1 The viral protein CrPV1A reveals a broad RNAi suppression activity among different species including *Tribolium*...71
5.2.1.1 Failed test of local suppression of CrPV1A: Experimental design or limitation of the technique? 71
5.2.1.2 CrPV1A has probably no effect on the microRNA pathway.............74
5.2.2 VP1 and CrPV1A reveal similar suppression mechanism but different potency 74
5.2.3 Potential reasons for the failure of FHV B2 and DCV1A........................75
5.2.4 Plant viral RNAi suppressors p38 and p25 might be ineffective in *Tribolium* due to their non-conserved interaction partners 76
5.2.4.1 Further plant VSRs might be more effective...................................77
5.2.5 Potential reasons for the failure of the Dicer-i line 77
5.2.6 The effectiveness of most RNAi suppressors remains unclear and needs further analyses 78
5.3 Establishment of a tool for temporal regulation of RNAi 79
5.3.1 Start of the RNAi effect after injection is too slow to separate sequential gene functions..79
5.3.2 Heat shock inducible CrPV1A activation: General considerations 80
5.3.3 Temporal RNAi regulation by CrPV1A activation is possible 81
5.3.4 Prospective application of hsCrPVi line to analyze genes involved in abdominal patterning 82
5.4 Concluding remarks 84
6 Literature 86
7 Appendix 106
7.1 Abbreviations...106
7.2 Primers used in this work 108
7.3 Identification of novel RNAi target genes in *Tribolium*...............................110
7.4 Single and double RNAi treatments of RNAi target genes 114
7.5 Phylogeny and GO term analysis 119
7.6 Repetition of *Tc-ebony* RNAi in transgenic inhibitor lines...........................126

7.7 Vector maps 127

8 Curriculum vitae 147

1 Summary

RNA interference (RNAi) is a highly conserved cellular mechanism and in the last years it has become to a major tool for functional analyses of genes in different species. Moreover, RNAi has recently been suggested as a novel and promising approach for pest control. Transgenic plants can be engineered to express double stranded RNAs (dsRNAs) targeting essential genes of a pest species. Upon feeding, the dsRNAs induce gene silencing in the pest, resulting in its death. However, the main challenge of RNAi-mediated plant protection is the identification of efficient RNAi target genes. In most pest species, the screening for RNAi target genes by a whole-animal-high-throughput-approach is not feasible due to missing genomic tools and limited breeding capacity.

Therefore, the first aim of this thesis was to use *Tribolium castaneum* (*Tc*) as a screening platform in order to identify the most efficient RNAi target genes. By employing the data from the *iBeetle* RNAi screen, some novel and highly efficient RNAi target genes were identified that induced organism death most rapidly after knockdown. The orthologs of these genes are hence excellent candidates for RNAi based pest control methods in other pest insects. Based on this set of RNAi targets, Gene Ontology term (GO term) combinations were identified that are predictive for efficient RNAi target genes and which detect proteasomal genes as prime targets. Further, I could show that the efficiency of RNAi mediated pest control cannot be increased by synergistic action in double knockdowns. Finally, an off target analysis revealed that protein sequence conservation does not strongly correlate with the number of potential off target sites, indicating that it will be difficult to design dsRNAs without potential off-target sites in non-target organisms.

In the second part of this thesis, I aimed to establish a method to regulate the RNAi response. Ubiquitous gene silencing can sometimes lead to pleiotropic effects, hampering the identification of specific phenotypes. Temporal and/or spatial regulation of RNAi can circumvent such effects. The viral RNAi suppressor protein CrPV1A can be used for this purpose. The expression of CrPV1A effectively blocks the RNAi mechanism and does not impair the development or viability of *Tribolium.* Probably, it also does not interfere with the microRNA (miRNA) pathway. Furthermore, temporal activation of CrPV1A is able to terminate the RNAi response of a previously silenced gene, resulting in a rescued RNAi phenotype. Temporally

controlled RNAi by CrPV1A activation is therefore an effective approach to investigate genetic interactions of further genes.

Parts of this thesis are being published in BMC Genomics: Ulrich, J., et al. (2015). Large scale RNAi Screen in *Tribolium* reveals novel Target Genes for Pest Control and the Proteasome as prime target.

2 Introduction

2.1 RNAi based strategies of plant protection

Worldwide, agriculture pests cause billions of dollars in crop losses by harming the plants or consuming grain or fruit products. Currently, the control of insect pests is mainly restricted to chemical pesticides, but this approach is becoming more challenging due to vast hazards to the environment and human health and the emergence of resistant pest populations, resulting in increasing costs of chemical plant protection (Ffrench-Constant, 2013, 2014; Moffat, 2001). An alternative strategy to keep pests under control is the application of genetically modified (GM) crops. Transgenic plants expressing *Bacillus thuringiensis* (Bt) insecticidal proteins belong to the common used GM crops. The soil-dwelling bacterium produces crystal protein toxins (Cry toxins) during sporulation, which attack midgut receptors of certain insect groups, resulting in insect death. Despite this specificity, some species have evolved resistance to Bt-toxins (Baxter et al., 2005; Gassmann et al., 2011; Gould et al., 1997; van Rensburg, 2007; Tabashnik et al., 2008, 2013). Furthermore, the fact that some important pests like leafhoppers, whiteflies and aphids are not affected by Bt proteins whereas non-target organisms can be harmed raised a demand for alternative and more specific transgenic approaches in plant protection (Dutton et al., 2002; Hilbeck et al., 1998, 1999; Losey et al., 1999). The most promising method for pest control appears the RNAi, which triggers sequence-specific silencing of gene expression upon injection or feeding of dsRNA (Gordon and Waterhouse, 2007; Narva et al., 2013; Price and Gatehouse, 2008). This evolutionary conserved mechanism was first observed in *Caenorhabditis elegans* (*Ce*) (Fire et al., 1998; Guo and Kemphues, 1995), in which dsRNA injection led to systemic degradation of specific messenger RNA (mRNA) and blocked gene expression in all cells as well as the offspring. Since then, it has become a major tool in functional analysis of genes in insects as well as a more specific alternative for plant protection (Akiyama-Oda and Oda, 2006; Brown et al., 1999; Bucher et al., 2002; Fire et al., 1998; Hughes and Kaufman, 2000; Liu and Kaufman, 2004; Meister and Tuschl, 2004; Pechmann et al., 2011; Scott et al., 2013; Winston et al., 2002).

2.1.1 RNA interference

RNAi is a universal gene-silencing mechanism in eukaryotic organisms. It is triggered by exogenous or endogenous double stranded RNA and regulates mRNA expression, silences transposons and defends cells against viral infections (Hammond, 2005; Meister and Tuschl, 2004). Upon entering the cell, dsRNA molecules are recognized and processed by the RNase III enzyme Dicer into 21-23 nucleotide (nt) long small interfering RNAs (siRNAs) (Bernstein et al., 2001; Elbashir et al., 2001). Subsequently, these siRNAs are loaded into the RNA-induced silencing complex (RISC) and unwound, resulting in an association between the single siRNA strand and the complex. The siRNAs serve as guides for RISC to find the complementary target mRNA, which is then cleaved after perfect base pairing (Fig. 2.1) (Hammond et al., 2000; Zamore et al., 2000). In *C. elegans* the RNAi mechanism is robust and long-lasting. This persistent response is accomplished by a two-step pathway. In the first step, dsRNAs are cleaved by Dicer into siRNAs. These siRNAs are, in the second step, incorporated into the RNA-dependent RNA polymerase (RdRP) which amplifies secondary dsRNAs, leading to a potent RNAi response (Sijen et al., 2001; Tomoyasu et al., 2008).

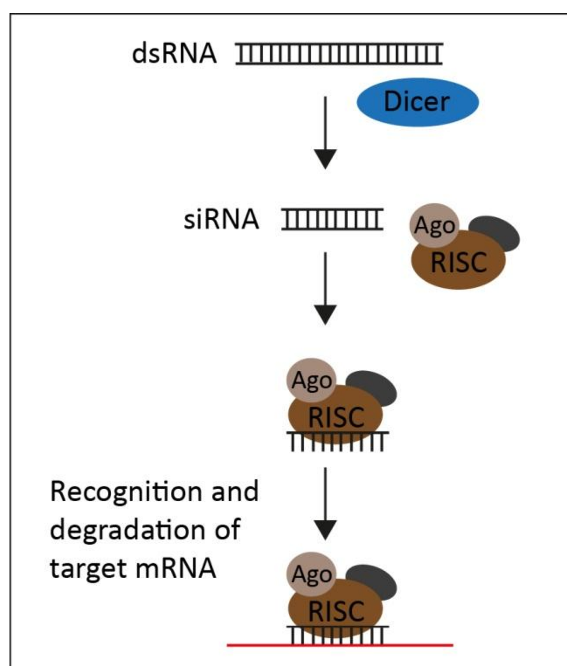


Fig. 2.1: RNAi mechanism.
The RNAi pathway is triggered by dsRNAs which are cleaved by Dicer into siRNAs. Subsequently, siRNAs are recognized and unwound by the RISC complex. RISC uses the single stranded siRNA as guide to find the target mRNA and to degrade it after perfect base pairing.

There are two conserved gene families that are universal components of the RNAi mechanism: the Dicer and the Argonaute family.

Dicer proteins (Dcr) involved in producing small RNAs reveal several conserved domains: two amino-terminal DExH-Box helicase domains, a Piwi/Argonaut/Zwille (PAZ) domain, tandem RNase III domains and a carboxy-terminal dsRNA binding domain (Bernstein et al., 2001; Carmell and Hannon, 2004; Tomoyasu et al., 2008). In *Drosophila melanogaster* (*Dm*) two different Dicer proteins, Dm-Dcr-1 and Dm-Dcr-2, are known to be responsible for two distinct processes. Dm-Dcr-1 specifically operates in the microRNA (miRNA) pathway which regulates the stability and rate of mRNA translation and thereby directs developmental processes. Dm-Dcr-2 is involved in the RNAi mechanism (Lee et al., 2004; Pasquinelli et al., 2005; Tomoyasu et al., 2008). In contrast to the situation in the fruit fly, *C. elegans* exhibits only one Dicer protein that is regulating both, the miRNA and the RNAi pathway (Bernstein et al., 2001; Ketting et al., 2001; Knight and Bass, 2001; Tomoyasu et al., 2008). Previous studies of the RNAi mechanism in *Tribolium castaneum* have shown that Tc-Dcr-2 is solely involved in the RNAi pathway, while Tc-Dcr-1 is suggested to participate in both processes (Tomoyasu et al., 2008).

Argonaute (Ago) proteins mediate target mRNA recognition as well as degradation and are core components of the RISC or the miRNA ribonucleoprotein particle (miRNP) complex, which is part of the miRNA machinery (Carmell et al., 2002; Meister and Tuschl, 2004; Parker and Barford, 2006; Tomoyasu et al., 2008). Ago proteins consist of two domains: a dsRNA binding PAZ and an RNAse H PIWI domain (Parker and Barford, 2006; Tomoyasu et al., 2008). Several Ago paralogs have been identified in *Drosophila* and *Tribolium*. *Drosophila* Dm-Ago-1 is involved in the miRNA and Dm-Ago-2 in the RNAi pathway (Okamura et al., 2004). Likewise, the recognized *Tribolium* Tc-Ago-1 is assumed to function in the miRNA mechanism, while two duplicated Tc-Ago-2 proteins have been shown to act in RNAi response (Tomoyasu et al., 2008). As in *Drosophila* and *Tribolium*, RNA mediated silencing processes of *C. elegans* use distinct Ago proteins for each mechanism: Rde-1 and Ergo-1 for the RNAi and Alg-1 and Alg-2 for the miRNA pathway (Tabara et al., 2002; Yigit et al., 2006).

In many species, including *Tribolium* and *C. elegans* the RNAi response spreads throughout the animal, resulting in systemic target gene silencing. For instance, *Tribolium* and *C. elegans* are even able to transmit the effect to the progeny (Bucher

et al., 2002; Liu and Kaufman, 2004; Lynch and Desplan, 2006; Sijen et al., 2001; Tomoyasu and Denell, 2004; Tomoyasu et al., 2008; Whangbo and Hunter, 2008). However, *Drosophila melanogaster* is known to miss such a robust and systemic effect and the genes responsible for these variations between several species still remain unclear. The presence of a *sid-1* gene, which is required for systemic spreading in *C. elegans* has been suggested to determine the presence or absence of systemic response in different organisms as *Drosophila* lacks *sid-1* (Roignant et al., 2003; Winston et al., 2002). Indeed, some insects, such as *Tribolium* and migratory locust (*Locusta migratoria*) reveal *sid-1-like* genes but these are not true orthologs of *Ce-sid-1* and appear not to be involved in systemic spreading (Luo et al., 2012; Tomoyasu et al., 2008). Hence, further analyses are necessary to completely understand the exact mechanism of robust, systemic RNAi response.

2.1.2 Application of RNAi in pest control and current limitations

RNA interference offers an opportunity to develop novel tools for pest control in agriculture. Due to the sequence specificity of RNAi, this mechanism might provide an outstanding approach to target individual or closely related species, excluding non-target organisms. Importantly, some insects are able to trigger RNAi response upon ingestion of dsRNA, which is a prerequisite for RNAi-mediated plant protection. The first report of gene silencing post feeding in insects was described in the light brown apple moth *Epiphyas postvittana* (Turner et al., 2006). Based on this, Baum et al. and Mao et al. have for the first time demonstrated that dsRNA expression of essential pest-specific genes in GM plants can trigger gene silencing in the western corn rootworm (WCR) *Diabrotica virgifera virgifera* and the cotton bollworm *Helicoverpa armigera*, resulting in insect death or developmental stunting upon oral dsRNA uptake (Baum et al., 2007; Mao et al., 2007). Furthermore, by feeding of WCR larvae with artificial diet supplemented with various dsRNAs against a large number of essential WCR genes, 14 efficient target genes were identified that induced mortality in the western corn rootworm even when provided low levels of dsRNA (Baum et al., 2007). Since then, these genes (mostly the midgut enzyme vacuolar ATPase (*vATPase*)) were used as targets for many other pest species in RNAi-mediated silencing approaches with varying success (Kwon et al., 2013; Li et al., 2013, 2011a, 2011b; Upadhyay et al., 2011). One major challenge of RNAi-

mediated plant protection is, hence, the effectiveness of target gene silencing. Sometimes, an efficient RNAi target in one species may be useless in another species. This could be due to varied susceptibility of different organisms to RNAi effects or to different target genes.

2.1.2.1 RNAi upon dsRNA ingestion

A core RNAi machinery is present in all insects (Gu and Knipple, 2013). Therefore, it is theoretically possible to target any pest by RNAi-mediated silencing of essential genes. However, some insects do not show a systemic RNAi response while others are not able to trigger RNAi upon dsRNA ingestion. For instance, dsRNA injection into larvae of the lepidopteran *Spodoptera litura* against a midgut aminopeptidase-N gene led to efficient transcript downregulation whereas ingestion of the same dsRNA produced no RNAi effect (Rajagopal et al., 2002). Hence, the success of RNAi-mediated pest control depends on the ability of the respective pest to trigger RNAi response.

2.1.2.2 Off targets

The specificity of RNAi based crop protection on target organisms is an important factor for the application of this technology in agriculture. Sequences that could affect non-target organisms (off targets) should be minimized. This could be accomplished, for example, by designing dsRNA constructs against less conserved gene fragments or against less conserved non-coding untranslated regions (UTRs) of mRNA transcripts which are responsible for mRNA transport, translation efficiency, subcellular localization and mRNA stability (Bashirullah et al., 2001; Jansen, 2001; Mignone et al., 2002; van der Velden and Thomas, 1999).

A further important aspect which should be considered when expressing RNAi target genes in transgenic plants is the emergence of unintended effects on plant physiology. A study in *Arabidopsis* has reported that transgenic RNAi plants revealed unexpected pleiotropic effects which resulted in reduced pollen viability, while no other obvious deviations from wild-type plant development were visible (Xing and Zachgo, 2007). This effect might alter plant fitness and biodiversity. Therefore, the potential for off targets in transgenic plants should also be carefully evaluated, for instance by searching for homologies between the RNAi target genes and the genome of the respective host plant (Fairbairn et al., 2007).

2.1.2.3 Endogenous plant RNAi mechanism

The development of transgenic plants capable of inducing RNAi response in insect pests was performed by plant transformation via *Agrobacterium tumefaciens* carrying vectors with inverted repeats of the target gene sequences (Baum et al., 2007; Kumar et al., 2012; Mao et al., 2007; Pitino et al., 2011; Zha et al., 2011). When transcribed in the plants they form hairpin RNAs, which are able to induce RNAi mechanism like long dsRNAs and thereby reduce transcript levels of respective genes. However, further investigations have shown that hairpin RNAs trigger plant RNAi response, resulting in cleaved siRNAs before being ingested by insects (Pitino et al., 2011; Zha et al., 2011). These short siRNAs caused less efficient silencing effects than long hairpin RNAs, indicating that the effectiveness of this strategy need to be increased (Kumar et al., 2012; Mao et al., 2007). Simultaneous downregulation of multiple targets has been done in several species, like *Drosophila*, *C. elegans* and *Rhipicephalus sanguineus* (Fuente et al., 2006; Schmid et al., 2002; Tischler et al., 2006) and might be an option to increase the RNAi effect in insects, which is hampered by endogenous plant RNAi machinery.

Furthermore, it has been reported that chloroplasts lack an endogenous RNA mechanism (Zhang et al., 2015). Thus, dsRNA expression in this cellular compartment can provide efficient crop protection.

2.1.2.4 Resistance development

RNAi-mediated plant protection techniques can be limited by potential emergence of sequence polymorphisms (small variations in DNA sequences) in the target gene of a pest species which might decrease the desired RNAi effect, resulting in resistance development to the RNAi-based control agent (Gordon and Waterhouse, 2007). Combinatorial expression of multiple target genes could help to minimize the possibility of resistance emergence. Likewise, successive expression of dsRNAs targeting different genes might be an option to overcome polymorphism development.

2.1.2.5 Target gene selection

The main limitation of RNAi-mediated plant protection is the identification of suitable target genes that will affect the pest after dsRNA digestion. Due to missing genomic and genetic tools and difficult rearing conditions in the lab, the screening for

RNAi target genes in agriculture pest species by a high-throughput-approach is not feasible. The main insect model system *Drosophila melanogaster* lacks systemic RNAi and is therefore not well suited to screen for RNAi target genes (Miller et al., 2008).

2.1.3 *Tribolium* as model system and screening platform

The red flour beetle, *Tribolium castaneum,* has developed to an excellent insect model organism in the last few years. It belongs to the most species rich insect order, the Coleoptera, and is a serious pest of stored grain products. In many respects the flour beetle development is more typical for insects than that of *Drosophila*. In contrast to the long-germ fly, *Tribolium* undergoes short-germ embryogenesis where the germ rudiment, composed of head and thorax anlagen, is build during blastodermal patterning. The posterior segments are produced successively from a posterior growth zone (GZ) during elongation. Besides, the presence of large extraembryonic tissues and unlike *Drosophila*, a non-involuted larval head are characteristic for *Tribolium* development (Bucher and Wimmer, 2005; Handel et al., 2000; Posnien et al., 2010; van der Zee et al., 2005). This mode of arthropod development is believed to be more ancestral and hence, results obtained from this beetle are more representative for insects in contrast to *Drosophila melanogaster* (Bucher and Wimmer, 2005; Klingler, 2004; Lynch and Roth, 2011; Schröder et al., 2008; Tautz et al., 1994).

Furthermore, *Tribolium* reveals a strong and systemic RNAi response, that can be elicited in any animal stage by dsRNA injection into the body cavity (Brown et al., 1999; Bucher et al., 2002; Tomoyasu and Denell, 2004). Moreover, dsRNA injection into female pupae or adults causes a long-lasting RNAi response in the offspring. In addition, by induction of RNAi in larval stages gene functions in post-embryonic and adult development can be analyzed (Tomoyasu and Denell, 2004).

Unlike the common pest species, *Tribolium* can be reared in large amounts in the laboratory. It reproduces all year round and reveals a short generation time. All these advantages make the flour beetle to a suitable organism for evolutionary and developmental biology. The accessibility to a sequenced genome and genetic and transgenic tools allow comprehensive functional analyses among others a large scale insertional mutagenesis screen (Berghammer et al., 1999; Richards et al., 2008;

Trauner et al., 2009). By transgenic tools, like heat shock and Gal4/UAS system, spatially regulated misexpression of genes is possible (Schinko et al., 2012, 2010).

Furthermore, an unbiased, large-scale RNAi screen (*iBeetle*) was performed in *Tribolium* (Schmitt-Engel et al., accepted). In this screen, more than 5.000 genes were investigated by pupal and/or larval RNAi. The resulting phenotypes were annotated and revealed a large number of novel genes with essential functions, including those that induced death of the injected animals most rapidly (Schmitt-Engel et al., accepted). The results are available on the iBeetle-Base (Dönitz et al., 2015).

2.1.4 Potential RNAi target genes

RNAi target genes for application in pest control are genes encoding proteins with essential functions. For example, arginine kinase (AK) is a phosphotransferase which is important in cellular energy homeostasis in invertebrates and is expressed in gut epithelial cells, muscle fibers as well as neurons (Chamberlin, 1997; Kucharski and Maleszka, 1998; Lang et al., 1980). It is only present in invertebrates and is assumed to be a promising candidate for pest control (Brown and Grossman, 2004; Liu et al., 2015; Pereira et al., 2000; Wu et al., 2007). Silencing of *AK* post dsRNA injection resulted in developmental disruption of the flea beetle, *Phyllotreta striolata* (Zhao et al., 2008). Likewise, expression of dsRNAs against the *AK* gene in transgenic *Arabidopsis* plants led to a drastic mortality rate of the insect pest, *Helicoverpa armigera,* upon plant feeding (Liu et al., 2015).

Similarly, downregulation of the vacuolar sorting gene *Snf7* which is involved in sorting and lysosomal degradation of transmembrane proteins, induced lethality in WCR larvae upon feeding with respective dsRNA (Baum et al., 2007; Ramaseshadri et al., 2013). Therefore, impairment of crucial protein functions might be helpful in controlling insect pests.

Further promising candidates for RNAi-mediated plant protection might be genes involved in proteasome assembly. The proteasome (26S proteasome) is a large protein complex composed of at least 32 different gene products (Wójcik and DeMartino, 2002). It consists of a proteolytic core particle, called 20S proteasome, and additional regulatory complexes (e.g. 19S complex) which bind either one or both ends of the core complex and thereby regulate its activity in various ways (Hölzl

et al., 2000; Peters et al., 1993; Yoshimura et al., 1993). The main function of 26S proteasomes or the ubiquitin-proteasome pathway is to catalyze degradation of cellular proteins in higher eukaryotes (Rock et al., 1994). This coordinated and temporal degradation of proteins by the ubiquitin-proteasome system influences cell cycle progression, transcriptional control and a vast number of other cellular pathways (Chen et al., 2005; Ciechanover, 1994; Hershko, 1997; King et al., 1996; Murray and Norbury, 2000; Spataro et al., 1998; Wang et al., 1998). Before degradation most substrate proteins are modified by polyubiquitin chains which allow the recognition of these proteins by 26S proteasome (Wójcik and DeMartino, 2002). Silencing of proteasome subunits in e.g. *Drosophila* S2 cells leads to reduced cell growth, increased apoptosis, reduced proteasome function and increased levels of ubiquitinated proteins, indicating inhibited protein degradation via the ubiquitin-dependent pathway (Heinemeyer et al., 1991; Soldatenkov and Dritschilo, 1997; Wójcik and DeMartino, 2002; Wójcik et al., 1996). In cancer therapy, disruption of the proteasome pathway by using inhibitors has already been successfully applied (Crawford et al., 2011). Proteasome inhibitors are able to specifically impair the proteasome function of cancer cells which are more susceptible to proteasome inhibitors than normal cells, resulting in cellular dysfunction and apoptosis (Almond and Cohen, 2002; Crawford et al., 2011; Ria et al., 2014; Wu et al., 2010). Furthermore, it has been demonstrated that knockdown of *Rpn7*, a non-ATPase subunit gene of the 26S proteasome, in the root knot nematode, *Meloidogyne incognita*, led to interrupted *M. incognita* locomotion as well as reduced egg production (Niu et al., 2012). Both *Rpn7* dsRNA soaking and dsRNA expression in transgenic plants significantly reduced nematode motility and infectivity and therewith suggested *Rpn7* as a promising target gene for controlling this plant parasitic nematode. Hence, this conserved multi-protein complex represents an excellent target for the application in pest control.

2.2 Aims

2.2.1 Identification of novel RNAi target genes for pest control

One aim of this work was to identify the most efficient RNAi target genes that induce organism death most rapidly after knockdown for their application in pest

control. Because pest species are usually too difficult to handle for a large scale screen, *Tribolium castaneum* was employed as a screening platform. The orthologs of these target genes are likely to represent good candidates for RNAi-mediated crop protection against other pest species, but this needs further investigations. In order to find those candidates the data produced by the large-scale unbiased RNAi screen *iBeetle* should be analyzed (Schmitt-Engel et al., accepted). For further selection, candidate dsRNAs need to be retested by titration experiments and verified based on their rapidly induced lethality. To examine whether the lethality can be increased synergistically, simultaneous downregulation of two dsRNAs, respectively, should be tested. GO term clustering analysis should identify GO term combinations that are predictive for potential RNAi target genes. Finally, an off target analysis should shed light on the species specificity of the candidate genes.

2.3 Temporal and/or spatial regulation of RNAi mechanism

Downregulation of endogenous genes via RNA interference has become a major role for the characterization of gene function in several organisms. However, due to the fact that genes are able to reveal a variety of functions throughout life cycle, constitutive and ubiquitous gene silencing can lead to pleiotropic effects which can hamper the correlation of phenotype to gene function. The possibility to initiate RNAi at different developmental stages in *Tribolium* can sometimes be an effective strategy to avoid such effects. For instance, RNAi in adult beetles might circumvent unwanted sterility which occurs after gene silencing during pupal development. However, a temporal shifting of RNAi is not able to prevent pleiotropy of genes that are involved in multiple functions simultaneously.

Pleiotropic effects were demonstrated e.g. for the segment polarity gene *wingless* (*wg*). Wingless is an essential protein for many processes in several species, including *Drosophila* and *Tribolium*. Among other functions, it is an important factor for leg development (Campbell et al., 1993; Cohen et al., 1993; Grossmann et al., 2009; Struhl and Basler, 1993). It has been tried to study its role in leg formation by using parental RNAi (pRNAi). However, *Tc-wg* dsRNA injection into pupae or adult beetles induced beetle sterility as well as a strong empty egg phenotype in case of some laid eggs, indicating multiple essential roles of *Tc-wg,* including gonad development or oogenesis. The same was true for early embryonic RNAi against *Tc-wg*. Silencing of *Tc-wg* expression either led to an empty egg phenotype or produced severely malformed cuticles where it was difficult to specifically analyze the leg phenotype (Grossmann et al., 2009).

A tool for temporally and/or spatially controllable activation of the RNAi mechanism would solve the problem of pleiotropic effects. In case of *Tc-wg,* a local knockdown of *Tc-wg* expression which is restricted e.g. to leg buds, would lead to a specific leg phenotype without other defects. Temporally controlled activation of *Tc-wg* RNAi might circumvent gonad and oogenesis defects which led to sterility, resulting in considerably more embryonic offspring with the RNAi effect. Moreover, the activation of *Tc-wg* silencing during later embryonic stages might rescue the early lethality phenotype, which was observed in RNAi embryos that were injected at the age of 4-8 hours (Grossmann et al., 2009).

In different species, transgenic systems are used to temporally and/or spatially regulate gene functions. For instance in mice, site-specific recombinase systems, like Cre/LoxP and FLP/FRT, has allowed to engineer spatial and temporal loss-of-function or gain-of-function mutations (Lobe and Nagy, 1998; Nagy and Rossant, 2001). Cre (Cyclization recombinase) or FLP (Flippase) proteins are able to induce DNA recombination if the target sequence is flanked by respective LoxP or FRT recognition sites. The orientation of LoxP sites determines whether target DNA will be cleaved or exchanged (Gierut et al., 2014). Furthermore, controlled RNAi mediated gene silencing is possible by expressing short-hairpin RNAs (shRNAs) against specific genes which are flanked by LoxPs (Coumoul et al., 2005; Kasim et al., 2004; Liao and Xu, 2008; Ventura et al., 2004; Xia et al., 2006). It has, for example, been demonstrated that mammalian cells, carrying shRNAs (with LoxP-Stop-LoxP sites in the loop of the shRNA sequence) driven by a tissue specific promoter, were not able to express shRNAs due to the LoxP-flanked Stop region which was placed within the shRNA fragment. When Cre recombinase was delivered to these cells, the Stop region was excised, resulting in spatial and temporal shRNA expression and therewith induced RNAi mechanism (Kasim et al., 2004).

In *Drosophila*, tissue-specific RNAi has been performed using the binary expression system Gal4/UAS (Dietzel et al., 2007; Fortier and Belote, 2000; Giordano et al., 2002; Martinek and Young, 2000; Piccin et al., 2001; Roignant et al., 2003). Besides, a genome-wide library of transgenic RNAi strains has been generated in which 88% of all predicted protein-coding genes can be targeted (Dietzel et al., 2007). To locally inactivate genes, the yeast transcriptional activator Gal4 was cloned downstream to a promoter that drives Gal4 in tissue-specific manner (Duffy, 2002). In addition, inverted repeats (IRs) of different target gene sequences were ligated downstream to the upstream activating sequence (UAS). Upon crossing the Gal4 driver line with the UAS responder line, Gal4 bound to the UAS site and thereby activated IR transcription in the pattern defined by Gal4, resulting in shRNA production and RNAi initiation. While these studies provide local control over RNAi, further methods have been developed that add temporal control to the Gal4/UAS system (Han et al., 2000; McGuire et al., 2003; Nicholson et al., 2008; Osterwalder et al., 2001; Roman et al., 2001). These either used a steroid-activated version of Gal4 or a temperature-sensitive Gal80^{ts} repressor to temporally and spatially regulate Gal4 expression. Steroid-activated Gal4 proteins were produced by

fusion of the Gal4 domain with a steroid hormone receptor domain, which was activated by respective ligand binding (Han et al., 2000; Nicholson et al., 2008; Osterwalder et al., 2001; Roman et al., 2001). For instance, the fusion protein of Gal4-progesteron-receptor could be activated by mifepristone (RU486) (Nicholson et al., 2008; Osterwalder et al., 2001; Roman et al., 2001), while the Gal4-estrogen-receptor fusion protein (Gal4-ER) was activated by exposure to estrogen (Han et al., 2000).

Alternatively, the Gal4/UAS system can be temporally regulated by Gal80^{ts}, a temperature-sensitive Gal4 repressor from *Saccharomyces cerevisiae*. Gal80^{ts} is able to repress Gal4 expression when animals are reared at 19°C and to activate Gal4 when shifting animals to 30°C (McGuire et al., 2003).

The binary Gal4/UAS system is also well established in *Tribolium* and has been shown to be a successful method for ectopic misexpression in this organism (Schinko et al., 2010). Furthermore, the Cre/LoxP system has been applied with promising results (Bucher, personal communication).

However, the main disadvantage of these introduced gene functional studies is that, additionally to the required tissue specific Gal4, Cre or FLP lines, each target gene has to be cloned separately and, therefore, a vast array of UAS, LoxP or FRT lines is required. Although genome-wide UAS-IR collections have been generated for *Drosophila* (Dietzel et al., 2007), this is not feasible for most other organisms due to the lack of balancer chromosomes.

2.3.1 Suppression of the RNAi pathway

The RNAi pathway is a natural antiviral defense mechanism in animal and plant cells. In order to overcome the host antiviral silencing pathway, some animal and most plant viruses have adopted counter defense strategies in form of RNAi suppressor proteins (Ding and Voinnet, 2007; Li et al., 2002; van Rij et al., 2006; Wang et al., 2006). These independently evolved viral suppressors of RNAi (VSRs) are able to target different steps of the RNAi pathway. Some of the VSRs sequester long dsRNAs or short siRNAs and thereby prevent their incorporation into RISC complex, while others directly interfere with Dicer or Argonaute proteins and impair the RNAi mechanism (Bortolamiol et al., 2007; Chao et al., 2005; Lakatos et al., 2006; Lu et al., 2005; Sullivan and Ganem, 2005; Vargason et al., 2003; Ye et al.,

2003; Zhang et al., 2006). Moreover, to increase viral pathogenicity some plant viruses are able to inhibit the miRNA pathway, resulting in disturbed host development, whereas some animal viruses have been demonstrated to interfere with the silencing mechanism directed against transposable elements (TEs) (Berry et al., 2009; Bortolamiol et al., 2007; Chapman et al., 2004; Dunoyer et al., 2004; Mérai et al., 2005; Yu et al., 2006).

Viral suppressor proteins have been exploited to study RNAi pathways in different organisms (Berry et al., 2009). Theoretically, VSRs could be used to temporally and/or spatially regulate RNAi silencing in developmental analyses, thus enabling gene functional studies in specific tissues or at certain time points. A requirement is, however, that VSRs only inhibit the RNAi mechanism and do not interfere with the miRNA pathway or produce developmental defects which would impede gene analyses.

2.3.1.1 FHV B2

The insect *Flock House virus* (FHV) belongs to the *Nodaviridae* family, which infects animals and plants in the natural environment. In order to ensure viral accumulation in the host, the positive-strand virus encodes an RNAi suppressor protein, B2, that binds long dsRNAs as well as siRNAs and thereby prevents their processing by Dicer and RISC proteins (Tab.2.1) (Chao et al., 2005; Li et al., 2002; Lingel et al., 2005). In addition, it has been demonstrated that B2 protein sequesters the PAZ domain of Dicer proteins in armyworm *Sf21* cells. Mutation in the C-terminus of B2 impaired its ability to inhibit RNAi and to bind Dicer proteins in these cells, suggesting that FHV B2 might suppress the RNAi mechanism by direct interaction with Dicer (Tab.2.1) (Singh et al., 2009). Furthermore, while FHV B2 seems not to interfere with the miRNA pathway in *Drosophila*, it has been shown to silence the suppression of TEs in somatic tissues and gonads, which might lead to increased viral infection in the fly (Berry et al., 2009).

2.3.1.2 DCV1A

Drosophila C virus (DCV) is a positive-strand virus from the *Dicistroviridae* family that is a major pathogen of many *Drosophila* strains and leads to a non-lethal persistent fly infection in nature (Aravin et al., 2003; Gomariz-Zilber and Thomas-

Orillard, 1993; Gomariz-Zilber et al., 1995; Thomas et al., 2003). The RNAi suppressor protein of DCV (DCV1A) specifically binds long dsRNAs and inhibits their Dicer-2 cleavage into siRNAs (see Tab. 2.1) (van Rij et al., 2006). Moreover, DCV1A has been shown to affect RISC complex assembly, suggesting an additional RNAi suppressor function (Nayak et al., 2010). Like FHV B2, DCV1A seems to suppress TEs silencing in armyworm *Sf21* cells but does not interfere with miRNAs or siRNAs (Berry et al., 2009; van Rij et al., 2006).

2.3.1.3 CrPV1A

Cricket Paralysis virus (CrPV) was initially identified and isolated from field crickets, *Teleogryllus oceanicus* and *Teleogryllus commodus,* and it is a highly potent virus of many species in the laboratory (Nayak et al., 2010; Plus et al., 1978; Reinganum et al., 1970). CrPV is closely related to *Drosophila C virus* and likewise belongs to the positive-strand *Dicistroviridae* family. In contrast to DCV, CrPV leads to mortality upon infection of crickets and flies (Manousis and Moore, 1987; Nayak et al., 2010). This high pathogenesis of CrPV is partially based on its efficient RNAi suppressor protein, CrPV1A (Nayak et al., 2010). It has been shown that adding CrPV1A to the Sindbis virus, which does not naturally encode an endogenous RNAi suppressor, resulted in increased virus production and fly lethality upon infection (Nayak et al., 2010). The mode of action of CrPV1A relies on its interaction with the endonuclease Ago-2, a component of the RISC complex (see Tab. 2.1). This interaction blocks Ago-2 cleavage activity, resulting in inhibited RISC-mediated mRNA degradation and therewith RNAi disruption. Nevertheless, the suppressor protein CrPV1A does not interfere with the miRNA pathway or alter the physiology and development of the animals when expressed in flies (Nayak et al., 2010).

2.3.1.4 Nora virus VP1

The positive-strand *Nora virus* is a member of the *Picornavirales* virus family and is a persistent natural pathogen of the fruit fly *Drosophila* (Habayeb et al., 2006). In order to establish virus infection and to counteract the host RNAi machinery, *Nora virus* has evolved a suppressor protein, the viral protein 1 (VP1) which is able to inhibit Ago-2 slicer activity, like CrPV1A (Tab.2.1) (van Mierlo et al., 2012). Although the viral suppressors, CrPV1A and VP1, do not show significant amino acid sequence similarities, both proteins reveal similar RNAi suppression mechanism,

indicating the important role of Ago-2 against viral infections (van Mierlo et al., 2012). Further analyses are necessary to investigate the effect of VP1 on the miRNA pathway and the animal development

2.3.1.5 TCV p38

The *Turnip Crinkle virus* (TCV) is a positive-sense RNA virus that belongs to the family of *Tombusviridae.* It was originally identified and isolated from turnip (*Brassica campestris* ssp. *rapa*) but TCV is able to infect a large number of plant species, including *Nicotiana benthamiana* and *Arabidopsis thaliana*. Like the most plant viruses, TCV also encodes a VSR, the p38, which is capable of disrupting plant RNAi mechanism by interacting with Ago-1 protein of plants. This interaction relies on the glycine/tryptophane (GW) domain of p38 that mimics host endogenous GW-containing proteins to recruit Ago proteins and therewith to assemble the RICS complex (Tab.2.1) (Azevedo et al., 2010). While plant Ago-1 protein is required for miRNA and siRNA-mediated target RNA degradation, the binding of p38 to Ago-1 does not seem to influence the miRNA pathway (Dunoyer et al., 2004; Ruiz-Ferrer and Voinnet, 2009; Schott et al., 2012). This might be due to distinct pools of miRNA- and siRNA-loaded Ago-1 proteins which are assumed to exist in plants (Schott et al., 2012). In order to analyze whether the RNAi suppression ability of p38 is transferable to insects, Berry et al. generated p38 expressing, transgenic *Drosophila* flies and treated them with exogenous dsRNAs (by injection) or endogenous siRNAs (by IR expression against a target gene). p38 in *Drosophila* transgenic lines did not reveal silencing activity, suggesting that interaction proteins of p38 are not conserved between plants and the fly (Berry et al., 2009). However, in animal cell cultures p38 production has been demonstrated to significantly interfere with the RNAi pathway (Dunoyer et al., 2004).

2.3.1.6 PVX p25

The *Potato virus X* (PVX, genus *Potexvirus*) is a positive-sense, single-stranded RNA virus that infects many plants, including species of the *Solanaceae* family. It encodes a multifunctional suppressor protein, p25, which is important for cell-to-cell movement of PVX and for inhibition of plant RNAi mechanism. The suppression activity of p25 partially relies on its ability to affect RNA-dependent RNA polymerase (RdRP) that amplifies dsRNA or siRNA/virus-derived RNAs (viRNAs) and thereby

enables a robust and systemic RNAi signal in plants as well as *C. elegans* (see Section 2.2) (Bayne et al., 2005; Schwach et al., 2005; Voinnet et al., 2000; Xie and Guo, 2006). Furthermore, p25 was also shown to interact with the nuclease Ago-1 and to promote its degradation (see Tab.2.1) (Chiu et al., 2010). Despite this interaction of p25 with Ago-1, it seems exactly like p38 not to affect the microRNA pathway. Furthermore, when expressing p25 in transgenic flies it was not able to suppress the RNAi mechanism (Berry et al., 2009; Dunoyer et al., 2004). Hence, the detailed suppression mechanism of p25 needs further investigation.

Tab. 2.1: Insect and plant viral RNAi suppressors.
Viruses with the appropriate RNAi suppressor proteins and their mode of action in the RNAi pathway.

Insect viruses encode viral suppressors of RNAi	**RNAi suppressor protein**	**Mode of action in animals**
Flock House virus	B2	Binds long dsRNA and siRNA; interacts with the PAZ domain of Dicer proteins
Drosophila C virus	DCV1A	Binds long dsRNA; affects RISC assembly
Cricket paralysis virus	CrPV1A	Interacts with AGO-2
Nora virus	VP1	Interacts with AGO-2
Plant viruses encode viral suppressors of RNAi	**RNAi suppressor protein**	**Mode of action in plants**
Turnip Crinkle virus	p38	Interacts with AGO-1 by the GW motif of the suppressor
Potato virus X	p25	Suppresses the host (RdRP)-dependent branch of RNA silencing; interacts with AGO-1

2.4 Aims

2.4.1 Establishment of a tool for temporal and/or spatial regulation of RNAi

A further aim of this work was to establish a tool for time and/or tissue-specific RNAi activation or repression. The binary Gal4/UAS system should be used to activate tissue-specific or ubiquitous expression of viral RNAi inhibitors which should repress the RNAi response in a temporal and/or spatial manner. To the same end, an

artificial RNAi inhibitor based on *Dicer-2* suppression by transgenic hairpin expression should be tested.

In order to gain temporal control over the RNAi effect the most potent RNAi inhibitor should be expressed via heat shock activation. By this inhibitor activation it should be tested whether previously silenced gene expression can be rescued.

3 Materials and Methods

3.1 Strains

Tribolium castaneum beetles were reared under standard conditions (Brown et al., 2009).The wild type strain *San Bernadino (SB)* was used for the RNAi target gene experiments.

The *vermillion white* (v^w) strain was used for transgenesis experiments. The strain revealed white eye color due to a mutation in the *Tc-vermillion* gene (Lorenzen et al., 2002a).

The transgenic beetle lines Bauchbinde-Gal4 (BB-Gal4), Boje-Gal4, Tc-hsp-EGFP [transgenic beetle line was generated by Johannes B. Schinko as described in (Schinko et al., 2012)], UAS-tGFP [transgenic beetle line was generated by Johannes B. Schinko as described in (Schinko et al., 2010)] and UAS-DsRed [transgenic beetle line was generated by Stefan Dippel (Dippel, personal communication)] used for the RNAi inhibitor experiments, were generated with piggyBac transgenesis in *vermillion white* strains. The enhancer trap lines BB-Gal4 and Boje-Gal4 were identified in an enhancer trap screen performed by Elke Küster in the lab. The mutator strain carries the transactivator piggyBac conctruct: pBac[3XP3-*Tc-vermillion*;Tc-bhsp-Gal4] marked with black eye color (generated by Johannes B. Schinko). The construct in this strain was remobilized by crossing with the M26 *jumpstarter/helper* strain (Lorenzen et al., 2007; Trauner et al., 2009), which carries an X-chromosomal insertion of a 3xP3-DsRed marked *Minos* element (pMi[3xP3-DsRed;Dm-hsp70-pBac] (Horn et al., 2003; Trauner et al., 2009). To visualize new insertions and potential novel enhancer trap patterns, the remobilized strain was crossed to a UAS-tGFP line. The resulting enhancer trap line BB-Gal4 shows Gal4 expression in a thoracic stripe pattern (called Bauchbinde or BB) in late larval, pupal and early adult stages. In early larval stages Gal4 reveals ubiquitous expression.

Except for early embryonic stages (0-24 h old) which do not reveal any Gal4 expression, the transgenic Boje-Gal4 strain appears to express Gal4 ubiquitously during all developmental stages.

3.2 Molecular cloning

Genes were amplified from complementary DNA (cDNA) of 0-72 h old embryos [cDNA was prepared by Sebastian Kittelmann and Jonas Schwirz with the SMART PCR cDNA Synthesis kit (Clontech)] with Phusion™ and cloned into pJET1.2 vector. Gene specific primers were either designed based on the AUGUSTUS gene predictions (version 4.0) and the transcriptome data at the *Tribolium* gene browser (http://bioinf.uni-greifswald.de/gb2/gbrowse/tcas4/) or based on the *Tribolium* sequences obtained from searching for orthologs with *Drosophila* gene sequences at the National Center for Biotechnology Information (NCBI, http://blast.ncbi.nlm.nih.gov/Blast.cgi) using the Basic Local Alignment Search Tool, BLAST (Altschul et al., 1990). Primers were generated using PCR primer designing software GeneRunner (www.generunner.com) and synthesized by Eurofins MWG Operon (Ebersberg, Germany). A complete list of primers is attached in the appendix (see appendix Tab. 7.1).

3.3 RNA interference

RNAi was performed using the established protocols (Posnien et al., 2009; Tomoyasu and Denell, 2004). Embryonic, pupal, larval and adult injections were performed with a FemtoJet® express device (Eppendorf, Hamburg, Germany) (and a borosilicate glass capillaries for embryonic injections) with an applied injection pressure of 150-300 hPa for embryos, 400-800 hPa for pupae and larvae (L6 instar larvae) and 500-1000 hPa for adult beetles. Injected embryos were allowed to develop for four days at 32°C under humid conditions and then were treated as described for cuticle analysis.

dsRNAs for the RNAi target gene experiments were ordered from Eupheria Biotech GmbH (www.eupheria.com), titrated from 0.5ng/µl to 1µg/µl and injected into 10 larvae or adult beetles, respectively. Titrated dsRNAs against the pigmentation gene *Tc-ebony* were injected in parallel as negative controls. For the best 40 target genes non-overlapping fragments (1µg/µl) were injected in additional RNAi experiments to rule out off target effects. Likewise, 1µg/µl concentrated dsRNA against *Tc-ebony* was used as negative control. The survival rate was scored every

second day post treatment. The respective primers and sequences are documented at iBeetle Base (Dönitz et al., 2015).

For the RNAi inhibitor experiments, dsRNAs were synthesized using the Ambion® MEGAscript® T7 kit (Life Technologies, Carlsbad, CA, USA). Templates for in *vitro transcription* were amplified by PCR using primers with an attached T7 polymerase promoter sequence (see appendix Tab. 7.2). The dsRNA concentration ranged from 1.6-2.7µg/µl and the injections were done at several developmental stages (see Tab. 3.1). The clone for *Tc-paired* dsRNA was provided by E. A. Wimmer lab (Developmental Biology, University Göttingen). The clones for EGFP and tGFP dsRNA were generated and provided by Johannes B. Schinko (Developmental Biology, University Göttingen).

Tab. 3.1: dsRNAs used for the RNAi inhibitor experiments.

The table shows the gene, the used dsRNA concentration, the fragment size and the injection method. (lRNAi: larval RNAi, pRNAi: pupal RNAi, eRNAi: embryonic RNAi).

Gene	dsRNA concentration (µg/µl)	Size (bp)	lRNAi	pRNAi	eRNAi
EGFP	2.6	720		X	
tGFP	2.5	780	X		
Tc-ebony	1.6	648	X		
Tc-paired	2.7	540		X	X

For cuticle analysis, developing L1 larvae were dechorionated in 50% Klorix bleach, embedded in 50 % Hoyer's medium 50 % lactic acid and were incubated at 65 °C overnight as described in (Bucher and Klingler, 2004). Cuticle defects were analyzed using a Zeiss Axioplan 2 microscope. Images were generated using the Cy3 filter set and the ImageProPlus (Media Cybernetics, Rockville, USA) software. Cuticle stack Z-projections were produced by recording 30 planes, deconvolving them by "No Neighbour" method and using the "Maximum Projection" method of ImageJ (Version 1.48, http://rsbweb.nih.gov/ij/disclaimer.html).

3.4 GO term clustering and identification of novel potential RNAi target genes

DAVID 6.7 (The Database for Annotation, Visualization and Integrated Discovery) (Huang et al., 2009a, 2009b) was used to analyse the eleven and 40 RNAi most efficient target genes. The *Drosophila* genome was set as background and p-values (p-value≤ 0.05) represented a modified Fisher's exact t-test. The enriched GO terms were clustered into classes by the functional annotation clustering tool, which uses a grouping algorithm based on the hypothesis that similar genes should share similar annotations. The option settings were: classification stringency "high" and enrichment thresholds "EASE 0.05". The enrichment score of each group is the geometric mean (in-log) of the p-values in an annotation cluster. Thus, the uppermost group shows the highest biological significance. GO Fat database, developed as part of the Annotation Tool of the DAVID suite of bioinformatics resources, was used for this analysis. This category filters out the broadest terms prior to the enrichment test so that they will not overshadow the more specific terms. The clustering of the top 40 RNAi target genes was done with 37 genes in total, because three genes were not associated with any GO term. Note that the last DAVID GO database update was in 2009, some GO terms have changed in the last years and could be wrongly annotated in the clusters. In order to find further potential RNAi target genes, the GO terms of each cluster were used in the Flybase Query Builder (Gelbart et al., 1997). With the obtained Flybase gene IDs we searched for *Tribolium* orthologs in the iBeetle Base (Dönitz et al., 2015). Genes that caused a mortality of ≥70% in the screen on day eleven after pupal or larval injection were assumed to be novel potential RNAi target genes.

3.5 Off target analysis

The nucleotide sequence of the RNAi target genes (Query) was used to identify potential off target sites in transcript sequences of other species by BLAST analysis. To this end, the length of the exact match was defined as ≥15 nt by the word size function at NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990) and the match/mismatch score was defined as 1/-4. Exact matches smaller than 17 nt were excluded manually.

3.6 Phylogenetic analysis

The *Tribolium* protein sequences were blasted against *Drosophila melanogaster*, *Apis mellifera*, *Aedes aegypti*, *Acyrthosiphon pisum* and *Mus musculus* RefSeq protein collection (Altschul et al., 1990) at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignments were done using ClustalW as implemented in the Geneious program (v.5.6.4) (Biomatters, Auckland, New Zealand) and trimmed to remove unclear parts of the alignments. The phylogenetic trees were calculated using the Geneious Tree Builder with the Jukes Cantor genetic distance model, neighbor-joining (Saitou and Nei, 1987) as tree building method, and a number of 10.000 replicates for creation of the bootstrap consensus tree (Felsenstein, 1985). Phylogenetic trees are attached in the appendix (see appendix Fig. 7.2).

3.7 Transgenesis

3.7.1 Constructs

All restriction enzymes were provided by Fermentas/Thermo Fisher Scientific (Waltham, MA, USA) or by New England Biolabs (Ipswich, MA, USA) and used according to manufacturer's protocols. Vector maps were designed with ApE – A plasmid Editor v2.0.47 (M. Wayne Davis; http://biologylabs.utah.edu/jorgensen/wayned/ape/) and are attached in the appendix (see section 7.7.). All primer sequences for construct design and sequencing are attached in the appendix (Tab. 7.3)

The plasmids for the viral suppressors of RNAi (VSR) CrPV1A (from *Cricket Paralysis virus*), FHV B2 (from *Flock House virus*), DCV1A (from *Drosophila C virus*) and VP1 (from *Nora virus*) were provided by Ronald Van Rij (Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, Nijmegen, the Netherlands). The plasmids for the viral suppressors of RNAi PVX p25 (from *Potato virus X*) and TCV p38 (*Turnip Crinkle virus*) were provided by Christophe Himber (Institut de Biologie Moléculaire des Plantes, UPR2357, CNRS, Strasbourg, France).

Plasmid pBac[3XP3-DsRed;UAS-Tc-bhsp-VSR-SV40] was generated as follows: The coding sequence of the RNAi suppressors was amplified via PCR with sequence specific primers (see Tab. 7.3). The PCR products were sub-cloned into the plasmid: pSLaf[UAS-Tc-bhsp-SV40] (generated by Johannes B. Schinko) using the restriction enzymes NotI and Acc65I. Finally, the UAS-Tc-bhsp-tGFP-SV40 fragment from the pBac[3XP3-DsRed] vector [generated by Johannes B. Schinko as described in (Schinko et al., 2010)] was exchanged with the UAS-Tc-bhsp-VSR-SV40 fragment from the pSLaf plasmid by AscI digestion and ligation.

Plasmid pBac[6XP3-ECFP;TcαTub1-3'UTRdcr2-intron-3'UTRdcr2-SV40] was generated as follows: The plasmid pBac[6XP3-ECFP] (generated by Johannes B. Schinko) was amplified via PCR using primers, JUFw_pBacL, JURv_Sv40, with attached restriction sites (see Tab. 7.3) and linearized by NheI and XhoI digestion. The ubiquitous *Tc-alpha-Tubulin1* promoter (843bp) (Siebert et al., 2008) was amplified via PCR from cDNA of 0-72 h old embryos using sequence specific primers, JUFwtubulin, JURvtubulin, with attached NheI and XhoI restriction sites (Tab. 7.3), cut and ligated with the *piggyBac* plasmid. The next cloning steps were done using the In-Fusion® HD Cloning kit (Clontech, Mountain View, CA, USA). The 3' untranslated region (3'UTR; 252bp) of *Tc-Dicer-2* was amplified by PCR with two primer pairs: UTRFw_Inf, UTRRv-Inf and UTR2Fw_Inf, UTR2Rv_Inf (Tab. 7.3) in order to obtain an inverted repeat (IR) orientation of this fragment. On the basis of reports about the ability of introns to enhance nuclear export (Llopart et al., 2002; Maniatis and Reed, 2002) an intron (931bp long) from a highly expressed *TC000503* gene was amplified via PCR from genomic DNA of adult *Tribolium* beetles with primers: IntronFw_Inf, IntronRv_Inf (Tab. 7.3). Genes were considered to be highly expressed if read coverage from RNA-Seq was ≥ 7000 (http://bioinf.uni-greifswald.de/gb2/gbrowse/tcas4/). The intron was ligated within the inverted repeats of 3'UTR *Tc-dcr2* to improve the stability during cloning (Kalidas and Smith, 2002; Lee and Carthew, 2003; Reichhart et al., 2002) and the fragment 3'UTRdcr2-intron-3'UTRdcr2 was ligated downstream of *Tc-αTubulin1* promoter into the linearized *piggyBac* vector (with NheI).

Plasmid pBac[3XP3-DsRed;Tc-hsp5'-CrPV-3'UTR-SV40] was generated as follows: The coding sequence of the RNAi suppressor CrPV1A was amplified using primers, JUFw_CrPVi and JURv_CrPVi (Tab. 7.3), with attached Acc65I and NotI restriction sites, cut and sub-cloned into the vector pSLaf[Tc-hsp5'-3'UTR]

(generated by Johannes B. Schinko). The Tc-hsp5'-EGFP-3'UTR fragment from the pBac[3XP3-DsRed] vector [generated by Johannes B. Schinko as described in (Schinko et al., 2012)] was replaced by Tc-hsp5'-CrPV-3'UTR fragment by AscI digestion and subsequently ligation.

3.7.2 Germline transformation and transgenic lines

Tribolium germline transformation was performed as described (Berghammer et al., 2009). All *piggyBac* constructs (500ng/µl) were injected along with a hyperactive helper plasmid [generated by Stefan Dippel (Yusa et al., 2011)] into embryos of the v^w strain. The constructs and hyperactive helper were mixed and filtered through a Millex-HV 0.45µm Filter Unit (Millipore, Billerica, MA, USA). Embryos were injected using a borosilicate glass capillaries and a FemtoJet® (Eppendorf AG, Hamburg, Germany). The embryos were kept under humid conditions for three days at 32°C and then transferred to lower humidity for hatching. The hatched larvae were transferred to whole grain flour and adult beetles (G0) were crossed to v^w strain.

Offspring (F1) were screened for red or blue fluorescent eyes and outcrossed with v^w strain. The heterozygous transgenic animals (F2) of this crossing were pooled and produced heterozygous and homozygous offspring. The established transgenic lines are summarized in Table 3.2.

Tab. 3.2: Established transgenic beetle lines.

RNAi suppressors and the established transgenic beetle lines are indicated. Superscript numbers of each VSR strain indicate different insertion lines. Except for hsCrPVi (i=inhibitor), a heat shock inducible inhibitor strain and Dicer-i line, a ubiquitous inverted repeat Dicer-2 strain, all transgenic beetles are UAS-VSR responder lines, which can be activated by a Gal4 driver strain.

RNAi suppressors	Established transgenic lines
CrPV1A	CrPVi^{56}, CrPVi^{61}, hsCrPVi^{47}, hsCrPVi^{49}
FHV B2	B2i^{22}, B2i^{40}
DCV1A	DCVi^{13}, DCVi^{21}
VP1	VP1i^{23}, VP1i^{20}
PVX p25	p25i^{30}, p25i^{35}
TCV p38	p38i^{55}, p38i^{25}
IR of dcr2-3'UTR	Dicer-i^{18}, Dicer-i^{22}

3.7.3 Transformation markers and GFP analysis

The 3 X P3 driven expression pattern of the fluorescent markers ECFP (Patterson et al., 2001) and DsRed1 (Horn et al., 2002) was detected in *Tribolium* eyes using a Leica MZ 16FA fluorescence stereomicroscope with a planachromatic 0.8 x objective (Leica, Wetzlar, Germany). The filter sets used for ECFP expression were: (425/50 nm excitation filter, 460nm LP emission filter). The filter sets used for DsRed1 were: (546/12 nm excitation filter, 605/75 nm emission filter).

The dark eye pigmentation of the *Tc-vermillion* marker (Lorenzen et al., 2002a) in the transgenic beetle lines BB-Gal4 and Boje-Gal4 could be observed without any filter.

The Tc-hsp5'-3'UTR driven ubiquitous expression of the fluorescent marker EGFP (Cormack et al., 1996; Yang et al., 1996) (transgenic beetle line Tc-hsp-EGFP) or the Gal4 driven expression pattern of the fluorescent marker tGFP in the "Bauchbinde" region was analyzed using the filter sets for EGFP: (470/40 nm excitation filter, 500 nm LP emission filter).

3.7.4 *Tribolium* genetic crosses

To activate RNAi suppressor expression by the binary expression system, transgenic pupae selected for the dominant markers of the driver (BB-Gal4 and Boje-Gal4 lines) and responder lines (CrPVi^{56}, CrPVi^{61}, B2i^{22}, B2i^{40}, DCVi^{13}, DCVi^{21}, VPi^{23}, VPi^{20}, p25i^{30}, p25i^{35}, p38i^{55}, p38i^{25}), respectively, were crossed together (G0). All crosses were carried out at 32°C. The F1 offspring of the crossings Boje-Gal4 X UAS-VSR or BB-Gal4 X UAS-VSR were examined to verify the presence of both driver (*Tc-vermillion* marker) and responder (DsRed marker) constructs. The crossings Boje-Gal4 X UAS-VSR were further treated with *Tc-ebony* dsRNA as described for RNAi.

The F1 offspring (heterozygous for both alleles) of the crossings BB-Gal4 X UAS-inhibitor were collected as pupae, selected for driver (black) and responder (red) eye markers and further crossed either to the heterozygous pupae of the line UAS-tGFP or Tc-hsp-EGFP. Line UAS-tGFP, line Tc-hsp-EGFP as well as UAS-VSR lines had DsRed as eye marker. The probability that F2 progeny of these crossings carried one copy each of the driver (BB-Gal4), the responder (UAS-VSR) and the responder

UAS-tGFP or Tc-hsp-EGFP allele was 12.5% (1/8), but it increased to 33.3% (1/3) (for the progeny of BB-Gal4 X UAS-VSR X Tc-hsp-EGFP) when selected for black and red eye markers. In the case of the crossing BB-Gal4 X UAS-VSR X UAS-tGFP the chance that F2 offspring carried all three alleles could be increased to 50% when selected for the eye markers and the obvious tGFP fluorescence in the BB. Subsequently, the selected pupae were injected with EGFP or tGFP dsRNA and/or heat shocked and analyzed for GFP fluorescence.

For the triple crossings, BB-Gal4 X UAS-VSR X Tc-hsp-EGFP, 300 pupae with black/red eye color were tested, respectively. The triple crossings BB-Gal4 X UAS-VSR X UAS-tGFP were carried out with 300 animals, respectively, that showed black/red eye color and tGFP fluorescence in the BB-tissue.

3.7.5 Heat shock conditions

Heat shock experiments were performed as described in (Schinko et al., 2012). The transgenic beetle line Tc-hsp-EGFP crossed to a Gal4 and UAS-VSR line was used to study the RNAi suppression. To this end, pupae were heat shocked for ten minutes at 48°C in a water bath to activate EGFP expression, one day post EGFP RNAi. EGFP knockdown was performed before heat shock mediated EGFP activation, in order to obtain complete downregulation of EGFP expression. Injected and heat shocked pupae were incubated for eight or twelve hours at 32°C and the EGFP fluorescence was documented. There were no significant differences in EGFP fluorescence between pupae analyzed for EGFP fluorescence 8 or 12 hours post heat shock. Due to that, further EGFP analyses were always done 8 hours post heat shock treatment.

In the case of embryonic heat shocks, transgenic line hsCrPVi (heat shock CrPV1A inhibitor line) was used. Staged embryo collections were allowed to develop for 10-15h, 11-16h and 12-17h at 32°C and heat shocked for two times at the same conditions as for pupal heat shocks. The regeneration time between the treatments was two hours at 32°C. Embryos were then allowed to develop into L1 larvae and were embedded and cuticles were analyzed as described for RNAi.

4 Results

4.1 Large scale RNAi screen identifies novel RNAi target genes

So far, the targets for dsRNA based pest control have been identified by small scale screens and on knowledge based approaches, i.e. by testing genes where previous data indicated an essential function. However, this approach will miss many genes that have not yet been linked to an essential function in one of the model species. Therefore, data produced by the large scale RNAi screen *iBeetle* (Schmitt-Engel et al., accepted) was screened, where randomly selected genes were downregulated by injection into pupae and larvae and the resulting phenotypes were documented in the iBeetle-Base (Dönitz et al., 2015). Of about 5,000 screened genes 100 revealed ≥90% mortality both nine days after pupal and eleven days after larval dsRNA injection (see appendix Tab. 7.4). In order to confirm these results and to test for sensitivity, different concentrations (3ng/µl, 30ng/µl, 100ng/µl, 300ng/µl and 1µg/µl) of the same dsRNAs were injected into 10 penultimate instar larvae (L6), respectively, and the survival rate was scored every second day. The pigmentation gene *Tc-ebony* was used as negative control. Injection of titrated *Tc-ebony* dsRNAs into larvae did not induce lethality in the treated organisms (Fig. 4.1 A). The most effective 40 genes caused a mortality of 50-100% at day eight post injection using the lowest concentrated dsRNA (see appendix Tab. 7.5, Fig. 7.1). I focused on the eleven most effective target genes, which were marked by mortality of 100% at least on day eight and of at least 80% on day six post injection (Fig. 4.1 B-M). This high degree of lethality was confirmed by repeating the experiment using non-overlapping dsRNA fragments (1µg/µl) making off target effects improbable (see appendix Tab. 7.7).

For comparison, the same experiment was performed with the orthologs of five RNAi target genes published in the seminal paper of Baum et al. (Baum et al., 2007), which caused lethality in the western corn rootworm upon dsRNA ingestion. Indeed, the *Tribolium* orthologs of these genes induced a high degree of mortality, but especially with low dsRNA concentrations, the mortality did not reach the one of the eleven candidates identified in this study (Fig. 4.1 N-R).

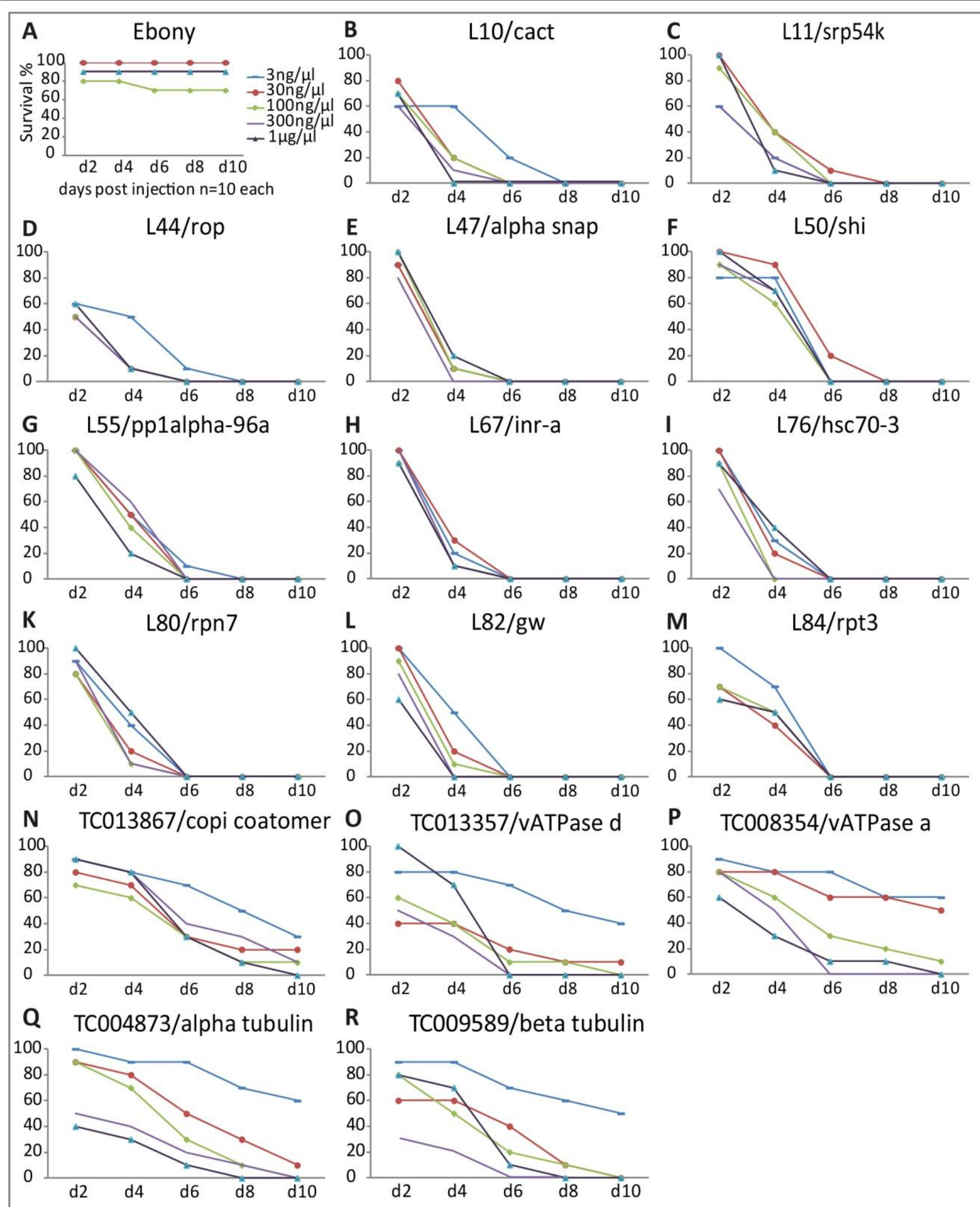


Fig. 4.1: Identification of novel RNAi target genes in *Tribolium castaneum.*

The survival after knock-down of the eleven most efficient RNAi target genes identified in this study is shown. dsRNAs of different concentrations were injected into 10 *Tribolium* larvae (L6 instar), respectively, and the survival rate was recorded every second day. (A) The pigmentation gene *Tc-ebony* was used as negative control. (B-M) Most of the identified RNAi target genes showed a larval mortality of 100% on day eight and 80% on day six post treatment for every dsRNA concentration. (N-R) RNAi treatment of commonly used targets based on the seminal paper of Baum et al., 2007 revealed a lower efficiency.

In order to check for stage dependence of the lethal effect, dsRNAs (concentration 100ng/µl and 3ng/µl) targeting these eleven genes were injected into 10 adult beetles, respectively. Likewise, titrated dsRNAs against *Tc-ebony* were injected as negative controls (Fig. 4.2 A). Mortality rate on day eight post treatment was at least 90% with 100ng/µl dsRNA concentration while injection of 3ng/µl dsRNA led to lower degree of mortality indicating that this concentration is at the lower limit for application (Fig. 4.2 B-M). In summary, eleven novel RNAi target genes were identified that efficiently and rapidly induce lethality at larval, pupal and adult stages even at low doses of dsRNA and are more efficient than previously used target genes at least in *Tribolium*.

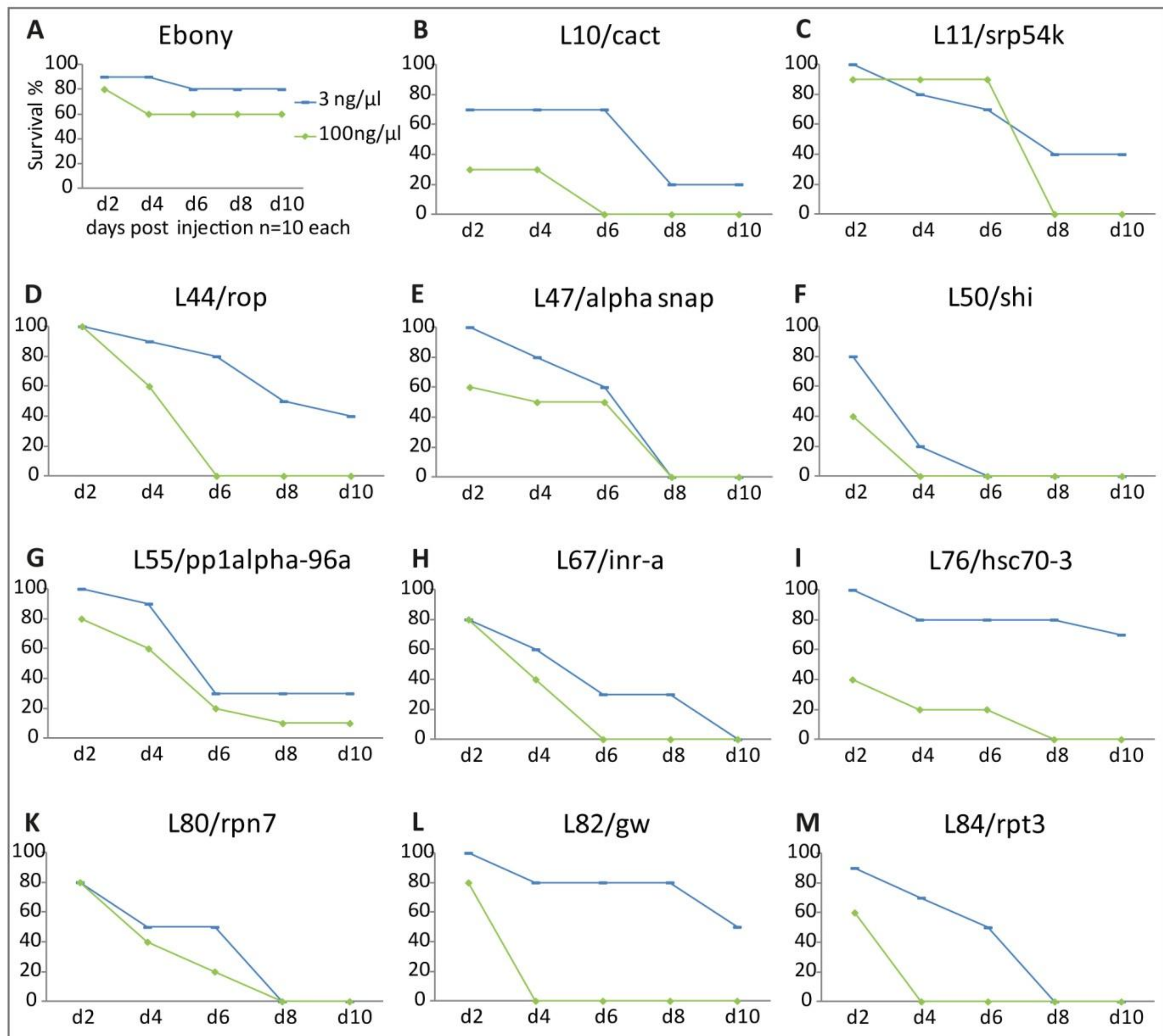


Fig. 4.2: Lethality is induced in adult stages as well.

The most efficient eleven RNAi target genes were also tested at two different concentrations (3ng/µl, 100ng/µl) by injections into adult beetles. (A) *Tc-ebony* was used as negative control. (B-M) All RNAi

target genes are lethal in adult stages as well but at lower concentrations, the efficiency was slightly lower compared to larval injections shown in Fig. 4.1.

4.1.1 Double RNAi led to additive but not to synergistic effects

I asked whether the lethality of RNAi treatments can be increased synergistically by combined injection of two dsRNAs targeting different essential genes. All 55 pairwise combinations of the eleven top RNAi target genes were injected into larvae (L6 instar) at the same dsRNA end concentration as the single injections (0.5ng/µl) and the survival was documented (see numbers in the appendix Tab. 7.8). *Tc-ebony* dsRNA (0.5ng/µl) was injected in parallel as negative control and did not induce larval mortality (see appendix Tab. 7.8). For better illustration of a potential synergistic effect, the survival differences between the double and the single knockdowns were calculated. For that purpose, the numbers of surviving animals of the single RNAis, e.g. L10 or L11, were subtracted from the numbers of surviving animals of the respective double knockdown, in this case of L10+L11 (Fig. 4.3 A-B). A higher lethality in the double treatment (e.g. L10+L11) should be indicated by negative values in both graphics e.g. of Fig. 4.3 A and B. There was no indication for synergism. Instead, the observed deviations from the baseline in some combinations are explained by additive effects: The most efficient targets become less penetrant when “diluted” with less effective dsRNAs (e.g. Fig. 4.3 A) while less efficient targets become more potent when supplemented with stronger target genes (e.g. Fig. 4.3 F). In conclusion, there was no indication for synergistic effects that would allow to significantly enhance the technique (Fig. 4.3).

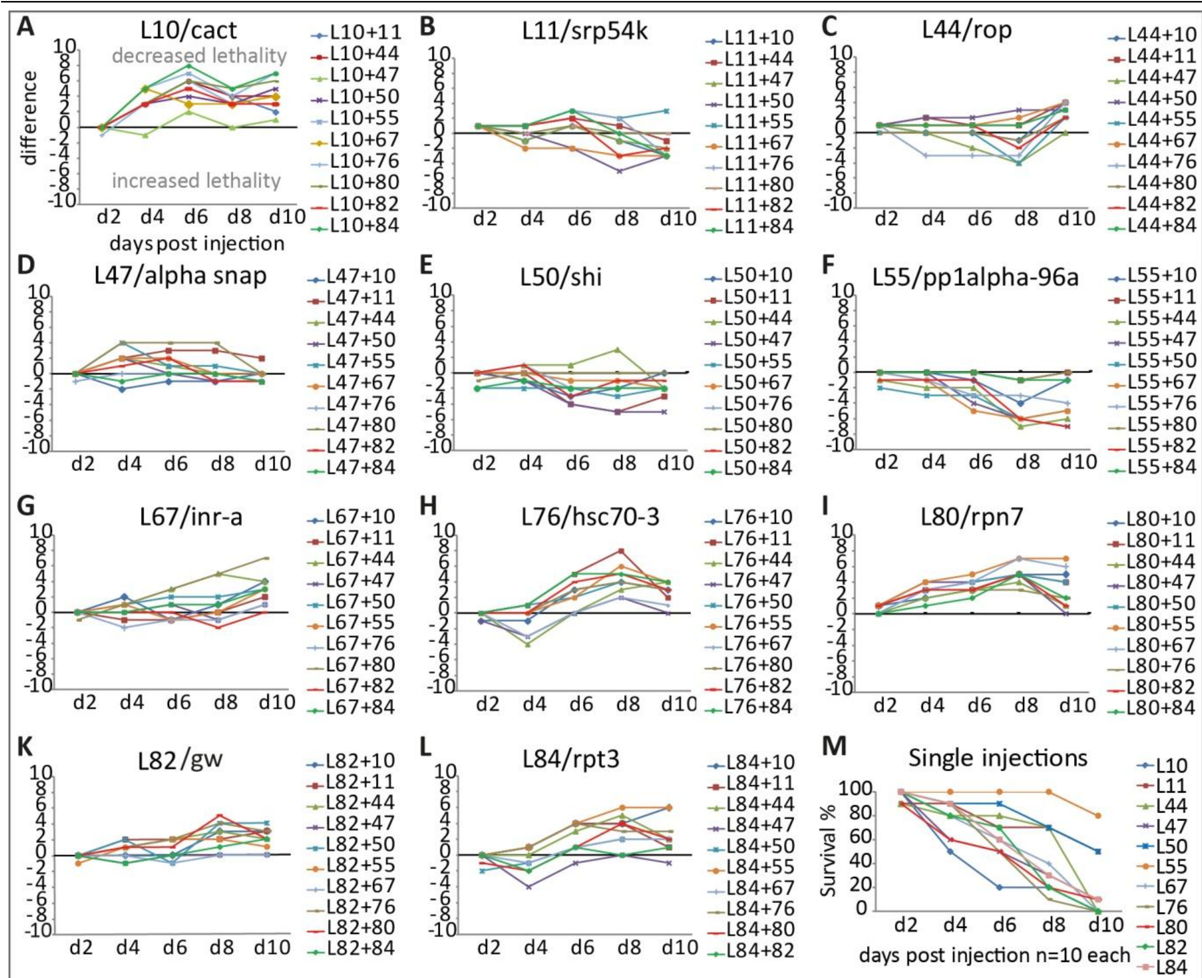


Fig. 4.3: Double RNAi led to additive but not synergistic effects.

(A-L) Double RNAi treatments were performed and the number of surviving animals at different days post injection was documented (see values in appendix Tab.7.8). From these values, the number of surviving animals of the respective single treatments (M) was subtracted, such that higher lethality in the double treatment is indicated by negative values in the panels. For every target gene, 10 different pairwise combinations were injected with a total dsRNA concentration of 0,5ng/µl and compared to the single injections (0,5ng/µl total concentration). (M) Single injections of the eleven RNAi target genes were performed with a dsRNA concentration of 0,5ng/µl.

4.1.2 Degree of sequence conservation does not strongly influence the number of off targets

In order to protect non-target organisms it would be desirable to use dsRNA fragments that are specific to the pest species and do not contain sequences targeting genes in non-target organisms (off targets). Therefore, I asked whether protein sequence conservation of the identified RNAi target genes correlated with the number of potential off target sites in other species. On the protein sequence level

most of the RNAi target genes showed a strong conservation between some well sequenced species covering insect diversity (*Drosophila melanogaster, Aedes aegypti* (Diptera), *Apis mellifera* (Hymenoptera), *Acyrthosiphon pisum (*Hemiptera). L10, L67 and L82 were the least conserved (Fig. 4.6 A). For protein L76 no ortholog could be identified in *Aedes aegypti* (Fig. 4.6 A; see appendix Fig. 7.2 H for phylogenetic analysis).

DsRNAs are processed by the enzyme Dicer into 21-23 nt long siRNAs. After incorporation into RISC, they serve as template to recognize the complementary mRNA and target it for destruction (Meister and Tuschl, 2004). However, siRNAs with an exact sequence identity of ≥17 nt can already induce off target effects (Kulkarni et al., 2006). Therefore, the nucleotide sequences of the eleven *Tribolium* target genes were searched against the well annotated NCBI transcriptome databases of the above mentioned species for ≥17bp long stretches of identity (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990). For visualization, these putative off target regions targeting genes of other species were plotted against the RNAi target gene nucleotide sequence (Fig. 4.4).

There was no overt correlation between the conservation of a protein and the number of potential off target regions (compare the most diverged genes L10, L67 and L82 with more conserved genes in Fig. 4.4). Likewise, within a given gene I found no enrichment of off target regions in more conserved stretches of the sequence (e.g. conserved protein domains) compared to less conserved stretches (e.g. non-coding UTRs; Fig. 4.4). Importantly, the location of off target sites was generally different for the different species. Together, these observations indicate that the number and location of the off target sites does not strongly correlate with protein sequence conservation.

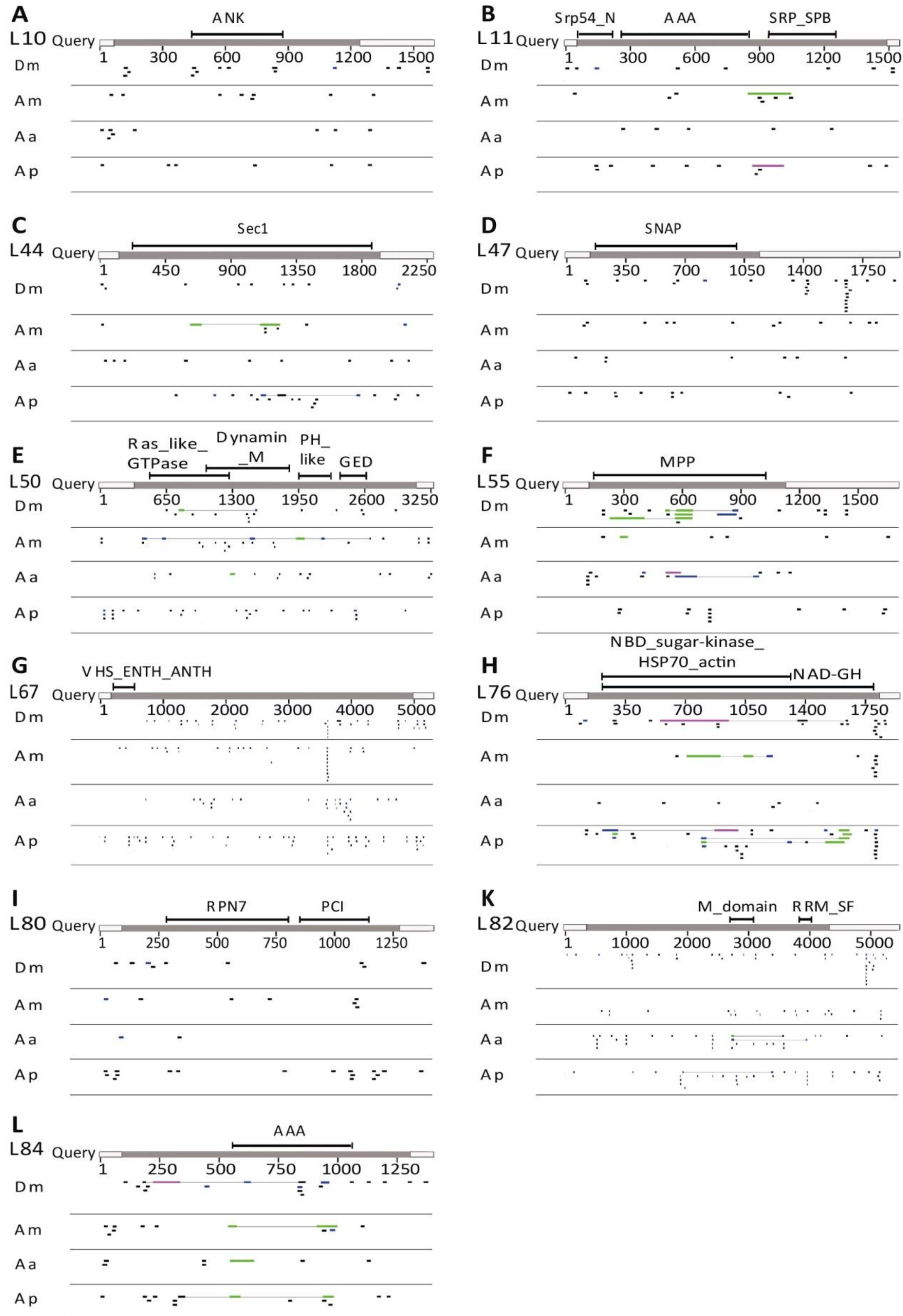


Fig. 4.4: Location of potential off target regions is not restricted to stretches of conserved protein sequence.

The non-coding and rapidly evolving UTRs are indicated as open bars, the coding sequence as grey bar. Conserved protein domains are indicated above the coding sequence by black bars and the

protein domain name. Sites with ≥17 nt identity were identified. The hits were plotted at the respective position of the query for each species (rows below the query). There was no strong if any correlation between the degree of sequence conservation (UTR<coding sequence<conserved domains) and the location of potential off target sites. This indicates that it will be difficult if impossible to exclude off target effects when misexpressing dsRNAs in plants. Multiple isoforms of different genes were not figured. Dm *Drosophila melanogaster*, Am *Apis mellifera*, Aa *Aedes aegypti*, Ap *Acyrthosiphon pisum.*

4.1.3 GO term clusters identify the proteasome as prime insecticide target

I tried to identify common properties of the identified RNAi targets, because this information might help identifying novel RNAi targets in species less amenable to large scale screens. I first analyzed the adult body expression levels in *Tribolium* and compared them to their *Drosophila* orthologs (Gelbart et al., 1997). A striking pattern was not found apart from generally high expression of the *Drosophila* orthologs, which was specifically true for the central nervous system (Fig. 4.5).

Tribolium Expression Level	L10	L11	L44	L47	L50	L55	L67	L76	L80	L82	L84
adult male body											
Drosophila Expression Level											
adult male body											
Drosophila Expression Level											
central nervous system, larvae L3											
salivary gland, larvae L3											
digestive system, larvae L3											
fat body, larvae L3											
carcass, larvae L3											

low expression | moderate expression | high expression | very high expression

Fig. 4.5: Expression levels of eleven RNAi target genes in *Tribolium* versus *Drosophila.*
Expression levels of the eleven RNAi target genes of *Tribolium* adult male body were obtained from RNA-Seq data of the *Tribolium* au2 gene set (http://bioinf.uni-greifswald.de/tcas/genes/au2/) and compared to the expression levels of the orthologous *Drosophila* genes in the adult male body obtained from modENCODE high-throughput RNA-Seq data in Flybase (Graveley et al., 2011). *Tribolium* expression levels were calculated and categorized into four expression strengths: low expression with 0-1.5, moderate expression with 1.6-3, high expression with 3.1-4.5 and very high expression with ≥4.6 number of reads per position. The comparison revealed similar expression levels only for L10, L11 and L76 in the adult male body of *Tribolium* and *Drosophila*.

Next, I searched for GO term clusters of the top eleven and top 40 RNAi targets (Huang et al., 2009a, 2009b) using *Drosophila melanogaster* GO term annotations as

the background. The clustering of the 40 RNAi target genes resulted in 10 clusters (see appendix Fig. 7.3). In order to test, in how far these GO terms are predictive, 1328 *Drosophila* genes were identified sharing the same GO term combinations of at least one of these clusters (see materials and methods for further details). For those, the respective *Tribolium* orthologs were determined and 502 of them had by chance been included in the *iBeetle* screen. Almost all novel genes identified by GO term combinations of cluster 1 and 2 (GO terms related to proteasome function) showed a strong lethality in the *iBeetle* screen (≥70% after pupal or larval injection; Fig. 4.6 B, C; see appendix Tab. 7.6). Cluster 7 (GO terms related to cytoskeleton organization) represented the third-best cluster with 37 of 66 novel genes showing lethality in the screen (Fig. 4.6 B, C; see appendix Tab. 7.6). The clustering of the top eleven RNAi target genes did not result in clusters, which were able to predict novel RNAi target genes. This could be due to the low number of input genes, which makes statistical analysis challenging (data not shown). Taken together, this analysis reveals GO term combinations that are predictive for potential RNAi target genes and identify the proteasome as prime target for insecticides.

A

Symbol	Tc-gene	Dm-ortholog	Dm gene name	Gene symbol	Dm	Am	Aa	Ap
L10	TC002003	CG5848	cactus	cact	25.3%	32.9%	29.1%	20.4%
L11	TC002574	CG4659	signal recognition particle protein 54k	srp54k	88.5%	88.2%	73.8%	87.2%
L44	TC011120	CG15811	ras opposite	rop	76.7%	76.4%	75.9%	76.3%
L47	TC013571	CG6625	alpha soluble NSF attachment protein	alpha snap	75.3%	84.2%	82.3%	73.6%
L50	TC011058	CG18102	shibire	shi	79.6%	86.0%	80.7%	82.5%
L55	TC015321	CG6593	protein phosphatase 1alpha at 96a	pp1alpha-96a	92.0%	94.8%	92.1%	92.1%
L67	TC008263	CG10228	inverse regulator a	inr-a	22.2%	33.9%	23.9%	25.0%
L76	TC004425	CG4147	heat shock 70-kDa protein cognate 3	hsc70-3	91.5%	90.4%	-	88.6%
L80	TC006375	CG5378	regulatory particle non-ATPase 7	rpn7	72.0%	74.6%	72.8%	37,8%
L82	TC006679	CG31992	gawky	gw	29.4%	39.3%	31.7%	36.1%
L84	TC007999	CG16916	regulatory particle triple-A ATPase 3	rpt3	91.7%	91.8%	92.9%	90.6%

B

Lethality of novel genes with clustering of Top40	Cluster 1 (gene number in % n=7)	Cluster 2 (gene number in % n=3)	Cluster 3 (gene number in % n=69)
70-100% lethality on d11	86% (6 of 7)	100% (3 of 3)	45% (31 of 69)
	Cluster 5 (gene number in % n=55)	**Cluster 6 (gene number in % n=243)**	**Cluster 7 (gene number in % n=66)**
70-100% lethality on d11	24% (13 of 55)	32% (77 of 243)	56% (37 of 66)
	Cluster 8 (gene number in % n=35)	**Cluster 10 (gene number in % n=19)**	
70-100% lethality on d11	49% (17 of 35)	42% (8 of 19)	

C

Annotation Cluster 1	Enrichment Score: 4,26			
Category	**Term**	**Count**	**%**	**PValue**
SP_PIR_KEYWORDS	proteasome	7	19	1,61E-09
GOTERM_CC_FAT	GO:0000502~proteasome complex	7	19	1,03E-06
KEGG_PATHWAY	dme03050:Proteasome	7	19	1,37E-06
GOTERM_MF_FAT	GO:0004175~endopeptidase activity	7	19	1,21E-02
GOTERM_BP_FAT	GO:0006508~proteolysis	8	22	2,39E-02
GOTERM_MF_FAT	GO:0070011~peptidase activity, acting on L-amino acid peptides	7	19	4,36E-02
Genes	CG16916, CG5378, CG18174, CG4157, CG4904, CG1782,CG4097, CG12323			
Annotation Cluster 2	**Enrichment Score: 2,37**			
SP_PIR_KEYWORDS	threonine protease	3	8	1,72E-03
INTERPRO	IPR001353:Proteasome, subunit alpha/beta	3	8	3,46E-03
GOTERM_MF_FAT	GO:0004298~threonine-type endopeptidase activity	3	8	5,05E-03
GOTERM_MF_FAT	GO:0070003~threonine-type peptidase activity	3	8	5,05E-03
GOTERM_CC_FAT	GO:0005839~proteasome core complex	3	8	9,76E-03
Genes	CG4904, CG4097, CG12323			
Annotation Cluster 7	**Enrichment Score: 1,92**			
GOTERM_BP_FAT	GO:0000226~microtubule cytoskeleton organization	6	16	6,31E-03
GOTERM_BP_FAT	GO:0007052~mitotic spindle organization	5	14	7,28E-03
GOTERM_BP_FAT	GO:0007051~spindle organization	5	14	1,21E-02
GOTERM_BP_FAT	GO:0007010~cytoskeleton organization	6	16	3,67E-02
Genes	CG8749, CG42341, CG4157, CG12323, CG7269, CG11522			

Fig. 4.6: GO term clustering reveals the proteasome as efficient target.

(A) All eleven RNAi target genes have *Drosophila* orthologs and overall, their protein sequence identity with other species is high. Only Cact, Inr-a and Gw show a low degree of sequence conservation. Dm *Drosophila melanogaster*, Am *Apis mellifera*, Aa *Aedes aegypti,* Ap *Acyrthosiphon pisum*. (B) GO term clustering of the top 40 RNAi target genes revealed ten clusters with enriched biological processes. Searching for genes that share the respective GO term combinations identified additional RNAi target genes. For cluster 1, seven novel genes with the respective combination were found and six of them (86%) turned out to be highly lethal at day 11 (d11) after injection (see appendix for genes Tab. 7.6). Cluster 1, 2 and 7 showed the highest predictive power. (C) The GO terms of the

most predictive clusters are shown. *Count* is the number of genes annotated with a given term. GOTERM_BP_FAT, GOTERM_MF_FAT, GOTERM_CC_FAT are annotations with respect to biological processes, molecular functions and cellular component (see appendix Fig. 7.3 for all clusters). See details for the enrichment score in materials and methods.

4.2 Identification of an efficient RNAi suppressor protein in *Tribolium castaneum*

The systemic RNAi response (i.e. the spread of injected dsRNA throughout the animal) in *Tribolium castaneum* can be obstructive for investigation of gene functions in certain tissues. Downregulation of genes with essential developmental roles can cause lethality or sterility in the injected animals masking their potential later functions in e.g. specific patterning processes. In order to circumvent such pleiotropic effects, a tool is necessary to restrict RNAi to a specific tissue or point in time. One possibility is the expression of viral proteins in *Tribolium*, which are known to suppress the RNAi machinery in different organisms. Once an efficient RNAi pathway suppressor is identified, controllable RNAi is possible by temporal or local activation of the suppressor. Hence, to identify efficient RNAi suppressors in *Tribolium*, my first approach was to investigate inhibitors previously tested in *Drosophila* and other organisms. The insect viral proteins from the *Cricket Paralysis virus* (CrPV1A), the *Flock House virus* (FHV B2), *Drosophila C virus* (DCV1A) and *Nora virus* (VP1) have been shown to work as suppressors of the RNAi mechanism in *Drosophila melanogaster* (Aliyari et al., 2008; Chao et al., 2005; Li et al., 2002; van Mierlo et al., 2012; Nayak et al., 2010; van Rij et al., 2006). Thus they were selected for analysis in *Tribolium.* Additionally, the plant viral proteins p25 from *Potato virus X* (PVX) and p38 from *Turnip Crinkle virus* (TCV) were tested, because of their known function to suppress the RNAi mechanism but not the microRNA pathway in plants (Bayne et al., 2005; Dunoyer et al., 2004; Jin and Zhu, 2010; Voinnet et al., 2000). In the following the viral proteins are referred to as RNAi inhibitors or VSRs (viral suppressors of RNAi). The different RNAi inhibitor strains are suffixed with an “i” (i=inhibitor).

4.2.1 VSRs did not suppress silencing of heat shock activated EGFP

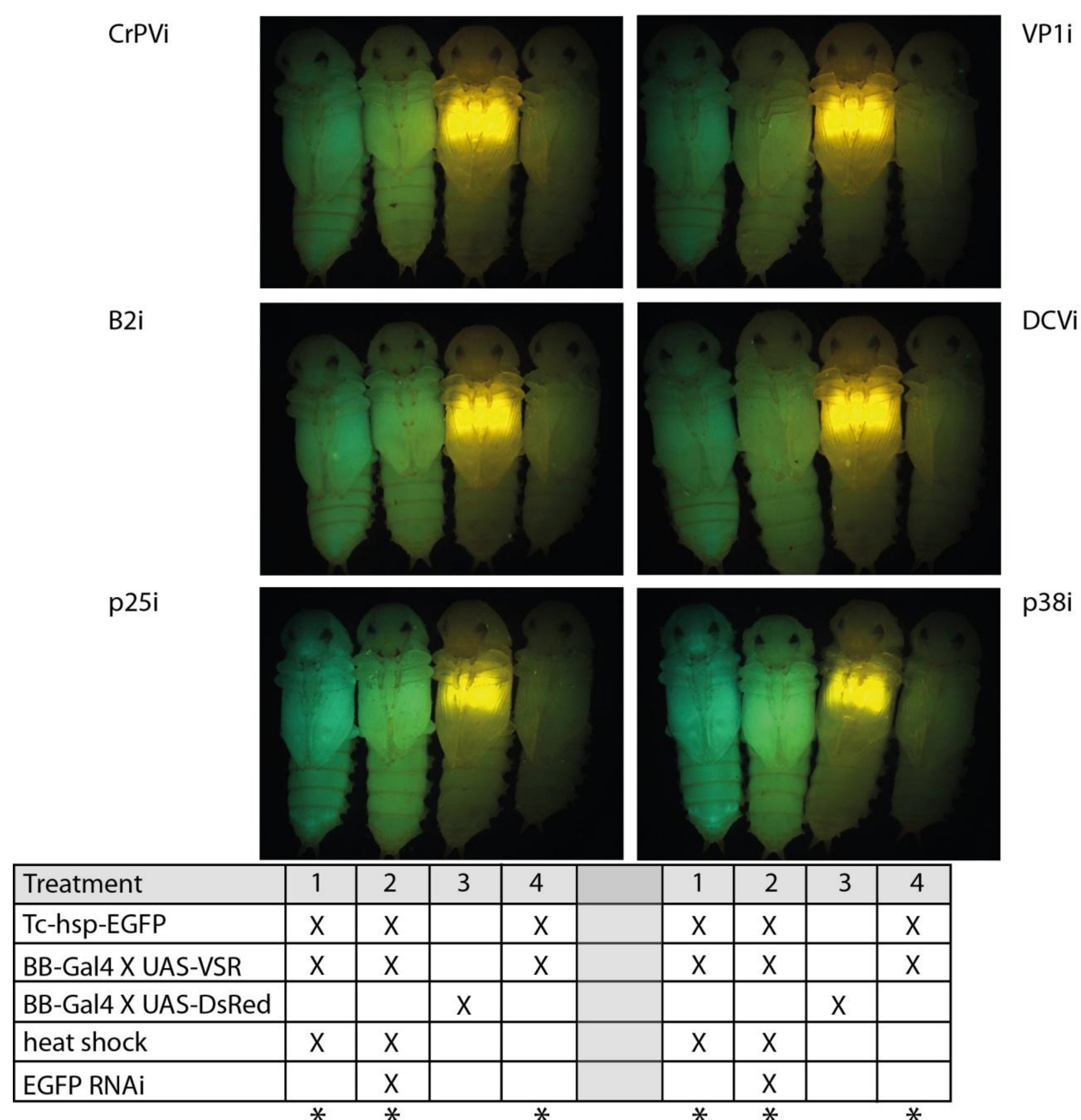
In order to test the suitability of the selected VSRs in *Tribolium*, each VSR was expressed in a restricted tissue and subsequently, a ubiquitously expressed target gene, the enhanced green fluorescent protein (EGFP), was silenced via RNAi. If a given VSR is able to suppress the RNAi pathway in *Tribolium*, EGFP signal should be gone after RNAi in all tissues except for those that expressed an active VSR.

For this rescue approach the binary Gal4/UAS system (Schinko et al., 2010) was used. The Gal4 line (marked by black eye color) shows Gal4 expression in a thoracic tissue (called “Bauchbinde” or “BB”) in late larval, pupal and early adult stages (Fig. 4.7, treatment 3, respectively, as a control for Gal4 expression). Upon crossing with UAS-VSR lines (marked by red eye color), VSRs were activated in the BB pattern. In order to exclude position effects, two independent transgenic lines for each of the six different VSR constructs were crossed to BB-Gal4 line, respectively. Subsequently, animals carrying both the driver BB-Gal4 and responder UAS-VSR were crossed to a Tc-hsp-EGFP strain (marked by red eye color) where EGFP was driven by an endogenous heat shock inducible promoter. This promoter leads to ubiquitous EGFP expression after heat shock at all developmental stages (Schinko et al., 2012). Because DsRed marked both the Tc-hsp-EGFP and the UAS-VSR construct, it was not possible to select for animals that carried all three vectors. Further, the Tc-hsp-EGFP and the UAS-VSR strains were heterozygous. Therefore, the chance that offspring of these crossings exhibited one copy each of the driver line (BB-Gal4), the responder UAS-VSR and Tc-hsp-EGFP was 33.3% when selected for black and red eye color (based on Mendelian inheritance). For that reason approximately 300 transgenic pupae (with black and red eye color) were treated with RNAi in each VSR test with the expectation that about 100 animals should contain all three constructs. Selected pupae were injected with EGFP dsRNA, heat shocked 24 hours post injection (see materials and methods) and then checked for EGFP fluorescence 8 hours later (Fig. 4.7). To ensure effective downregulation of EGFP, RNAi was performed before heat shock mediated EGFP activation and tests revealed that EGFP expression could be completely silenced after this treatment (data not shown). As a positive control 100 transgenic pupae (black/red eye color) were heat shocked but not injected with dsRNA and around 60 revealed a heat shock inducible EGFP fluorescence (Fig. 4.7, treatment 1, respectively).

I could not detect any GFP fluorescence in the BB-region where expressed VSRs should suppress the RNAi mechanism resulting in persistent EGFP signal (Fig. 4.7, treatment 2, respectively). All six tested lines (with two different insertion lines, respectively) showed no significant differences to each other and the negative control which was neither injected nor heat shocked (Fig. 4.7, treatment 4, respectively). Furthermore, the analysis of EGFP fluorescence after downregulation did not reveal significant differences among the injected animals of the same crossing experiment,

despite the fact that only one third of the transgenic pupae carried one copy of all three constructs while the remaining two third missed either Tc-hsp-EGFP or UAS-VSR.

A possible explanation for the failure of this test might be that the potentially rescued EGFP fluorescence in the BB could be insufficient to be apparent. Especially, since the pupal wings are known to exhibit autofluorescence it is possible that they would mask weak BB-EGFP signal. Hence, a clearly visible signal is necessary to monitor effective RNAi suppression.



Treatment	1	2	3	4		1	2	3	4
Tc-hsp-EGFP	X	X		X		X	X		X
BB-Gal4 X UAS-VSR	X	X		X		X	X		X
BB-Gal4 X UAS-DsRed			X					X	
heat shock	X	X				X	X		
EGFP RNAi		X					X		
	*	*		*		*	*		*

Fig. 4.7: Test of different VSR lines for the ability to suppress the RNAi mechanism.
Six different VSRs were tested in *Tribolium.* Anterior is up for pupae. Ventral views. Treatment 1-4 indicates that the pupae of the respective treatment carried the same constructs and were treated in the same way. The pupae of treatment 1, 2 and 4, respectively, carried the BB-Gal4, the respective

UAS-VSR and the Tc-hsp-EGFP construct (see table).The pupae of treatment 3, respectively, carried Gal4 and UAS-DsRed construct and served as a control for BB-Gal4 expression. Stars indicate that only one third of the pupae in the respective column carried all three constructs (see text for further details). The pupae of treatment 1, respectively, served as positive controls for fluorescence and were heat shocked but not injected (see table for treatments). Each pupa of treatment 4 served as a negative control for fluorescence and was neither heat shocked nor injected. The pupae of treatment 2, respectively, were injected with EGFP dsRNA (2.6 µg/µl) and heat shocked to activate ubiquitous EGFP expression (see table for treatments). Pupae with an efficient RNAi inhibitor should exhibit BB-restricted EGFP fluorescence. However, there were no visible fluorescence differences to the controls (compare each second pupa to each fourth control pupa; fourth pupa appears darker due to the lateral position in the picture). Due to the same results between several insertion lines, only one VSR insertion line for each UAS-VSR construct is shown: CrPVi[56], B2i[22], DCVi[13], VPi[23], p25i[30], p38i[55] (i=inhibitor) (see materials and methods).

4.2.2 CrPV1A efficiently suppressed the knockdown of UAS-Gal4 expressed tGFP

To ensure a strong fluorescence in the rescue VSR experiments, BB-Gal4 (black eye marker) was crossed to a responder line, UAS-tGFP (red eye marker), which revealed an obvious GFP fluorescence in the BB when activated by Gal4 (Fig. 4.8, treatment 1, respectively). The progeny of this crossing (black/red eyes) were further crossed to UAS-VSRs (red eye marker). Thus, VSRs and GFP were co-expressed in the same BB-tissue (Fig. 4.8, treatment 3, respectively).

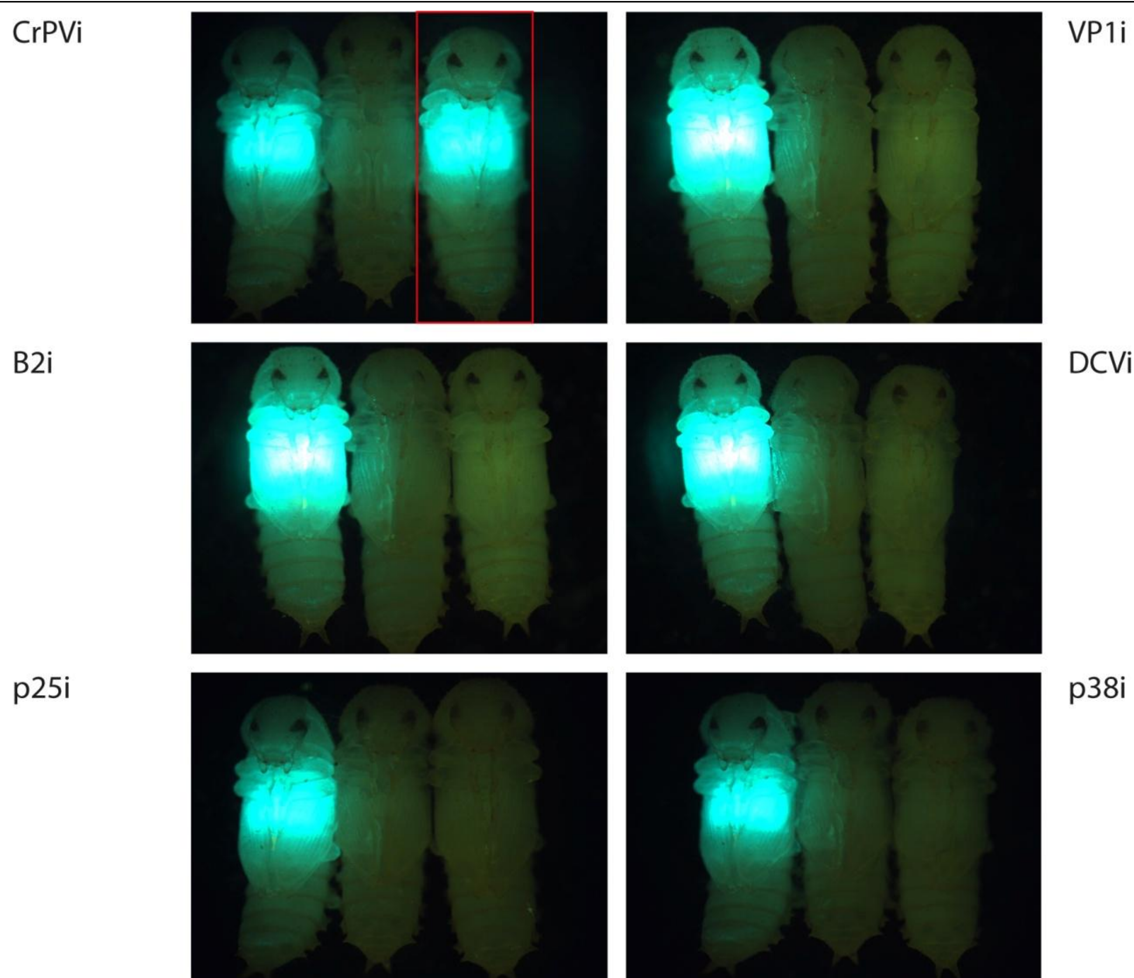
The activation of VSRs in the same tissue as turboGFP (tGFP) should locally suppress the RNAi mechanism and thus rescue BB-tGFP expression upon tGFP knockdown, if a given VSR is able to inhibit RNAi in *Tribolium*.

Again, six transgenic UAS-VSR lines, with two different insertion lines, respectively, were tested. Due to the same DsRed eye marker in the strains, UAS-tGFP and UAS-VSRs, two different selection criteria were applied. First, the larval progeny of these crosses were screened for black/red eye color. Second, only larvae were selected that revealed BB-tGFP fluorescence. These criteria increased the chance that the heterozygous animals carried all three constructs to 50% (based on Mendelian inheritance). The remaining 50% carried one copy of BB-Gal4 and one copy of UAS-tGFP without UAS-VSR, which could additionally be used as an internal positive control for tGFP silencing post injection. Each VSR approach was performed with approximately 300 transgenic larvae, of these about 150 were expected to

contain all three constructs. L6 instar larvae were injected with tGFP dsRNA (see materials and methods), incubated for one week at 32°C until pupation to allow decay of tGFP protein and then analyzed for GFP fluorescence (Fig. 4.8, treatment 3, respectively). In the control, tGFP RNAi of transgenic larvae, carrying BB-Gal4 and UAS-tGFP led to complete downregulation of the tGFP signal (Fig. 4.8, treatment 2, respectively). Untreated BB-Gal4 X UAS-tGFP animals served as negative controls (Fig. 4.8, treatment 1, respectively).

Of the tested VSR lines, only transgenic BB-Gal4 X UAS-CrPVi X UAS-tGFP line revealed GFP fluorescence in the BB-region after tGFP knockdown (Fig. 4.8 pupa in the red box). This was true for both insertion lines of UAS-CrPVi (CrPVi^{56} and CrPVi^{61}) (data not shown). About half of the treated animals (141 pupae of insertion line CrPVi^{56} and 134 pupae of insertion line CrPVi^{61}) revealed continuous BB-GFP fluorescence after RNAi, while in the remaining animals (138 pupae of CrPVi^{56} and 127 pupae of CrPVi^{61}) tGFP expression was completely silenced. Inefficient silencing could therefore be excluded. These results coincided with the expectation that 50% of the selected transgenic animals would carry all three constructs.

In summary, this approach could identify the protein CrPV1A from *Cricket Paralysis virus* as an efficient RNAi suppressor in *Tribolium castaneum*.



Treatment	1	2	3		1	2	3
BB-Gal4 X UAS-tGFP	X	X	X		X	X	X
BB-Gal4 X UAS-tGFP X UAS-VSR			X				X
tGFP RNAi		X	X			X	X

Fig. 4.8: CrPV1A was able to suppress the RNAi pathway in *Tribolium castaneum.*

Six transgenic VSR lines were analyzed for the ability to inhibit RNAi in *Tribolium.* Anterior is up for pupae. Ventral views. Treatment 1-3 indicates that the pupae of the respective treatment carried the same constructs and were treated in the same way. The pupae of treatment 1 and 2, respectively, carried a Gal4 and UAS-tGFP construct (see table). Each pupa of treatment 3 is a crossing result of Gal4, UAS-tGFP and the respective UAS-VSR line (see table). The pupae of treatment 1, respectively, are RNAi untreated pupae (see table for treatment) which served as negative control for RNAi and revealed a strong BB-specific tGFP expression. The pupae of treatment 2, respectively, served as positive control for RNAi and showed complete tGFP silencing after tGFP dsRNA (2.5µg/µl) injection (see table for treatment). The pupae of treatment 3, respectively, were injected with dsRNA against tGFP (2.5µg/µl) and represent the VSR rescue test. Only CrPVi line revealed efficient RNAi pathway suppression and displayed BB-tGFP fluorescence despite tGFP silencing (pupa in the red box). Due to the same results between several insertion lines, only one VSR insertion line for each UAS-VSR

construct is shown: CrPVi[56], B2i[22], DCVi[13], VP1i[23], p25i[30], p38i[55] (i=inhibitor) (see materials and methods).

4.2.3 Confirmation of CrPV1A as an efficient inhibitor

In order to confirm the results from the previously described VSR approach (see section 4.2.2) a further test was performed, in which downregulation of a target gene should be rescued by the respective VSR expression. For that purpose, the *Tribolium* pigmentation gene *Tc-ebony* was used as target gene. *Ebony* codes for a NBAD (N-beta-alanyl dopamine) synthetase, which is important for the formation of NBAD sclerotin and is known to be involved in body color formation in different insects (Hopkins and Kramer, 1992; Wright, 1987). Larval RNAi against *Tc-ebony* in the wild type (wt) *SB* strain led to darkening of the adult body color from red-brown to black (Fig. 4.9 A-B). In animals, a blocked RNAi pathway due to ubiquitous VSR expression should result in wild type body colored beetles despite *Tc-ebony* knockdown.

For this VSR approach, UAS-VSR lines (red eye color) were crossed to a Boje-Gal4 strain (black eye color), an enhancer trap line which appears to express Gal4 ubiquitously during all developmental stages except for early embryos (0-24h) (see materials and methods). Larval progeny of these crossings were screened for black and red eye markers and subsequently treated with dsRNA against *Tc-ebony*. Injected animals (L6 instar larvae) were incubated for approximately three weeks at 32°C until hatching and then analyzed for body color development. This treatment confirmed that the *Cricket Paralysis virus* protein, CrPV1A, is an efficient RNAi suppressor. Both CrPVi insertion lines revealed a wild type or dark-brown body color phenotype despite *Tc-ebony* downregulation, indicating that the RNAi machinery was effectively blocked (Fig. 4.9 C-D). *Tc-ebony* RNAi in transgenic lines with an active FHV B2 protein (B2i[22] and B2i[40]) resulted in a diverse body color phenotype with 46% black colored, 25% dark-brown colored and 29% wild type colored animals for B2i[22] (Fig. 4.9 E) and 57% black colored, 14% dark-brown colored and 29% wild type colored animals for B2i[40] (Fig. 4.9 F). This indicated some activity in suppressing of RNAi. Nevertheless, FHV B2 was excluded from further analysis due to insufficient RNAi inhibition in the previous rescue experiments (Fig. 4.7 and Fig. 4.8). The remaining VSRs were not able to interfere with the RNAi mechanism in my tests and, hence, were excluded from further study, as well (Fig. 4.9 G-O).

To avoid false positive results, I repeated the experiments, including positive controls which confirmed efficient *Tc-ebony* knockdown and achieved the same results for the analyzed VSRs (see numbers in the appendix Tab. 7.9).

4.2.4 Silencing of endogenous *Dicer-2* gene failed to suppress *Tc-ebony* RNAi

In order to test whether silencing of the *Tribolium* gene *Tc-Dcr-2* which encodes a protein involved in the RNAi pathway would lead to the same strong RNAi inhibition as described for CrPV1A, I designed a further transgenic line carrying inverted repeats against *Tc-Dcr-2* (Tomoyasu et al., 2008). IRs have been reported to induce RNAi effect in *Drosophila* (Fortier and Belote, 2000; Kennerdell and Carthew, 2000; Lam and Thummel, 2000; Lee and Carthew, 2003; Martinek and Young, 2000) and IR-mediated *Tc-Dcr-2* depletion should ensure RNAi suppression.

The 3' UTR of *Tc-Dcr-2* mRNA was used as IR target. To enhance nuclear export of the transgene (Llopart et al., 2002; Maniatis and Reed, 2002) and exclude cloning difficulties (Kalidas and Smith, 2002; Lee and Carthew, 2003; Reichhart et al., 2002) a functional intron was placed between the IRs and the entire fragment was cloned downstream of the ubiquitously and constitutively active *Tc-alpha-Tubulin1* promoter (Siebert et al., 2008) (see materials and methods). Upon mRNA expression of the transgene, it should form hairpin-loops and induce RNAi response leading to competitive inhibition of the endogenous *Tc-Dcr-2* mRNA translation in *Tribolium*. Like the viral VSR strains, two independent transgenic insertion lines for this construct (called Dicer-i^{18} and Dicer-i^{22}) were tested for the suitability to interfere with the RNAi mechanism.

Larval offspring of the Dicer-i strains (blue eye marker) were screened for the eye marker and subsequently treated with dsRNA against *Tc-ebony* as described in section 4.2.3. However, both Dicer-i insertion lines did not show obvious RNAi suppression after RNAi (Fig. 4.9 P-Q). The same was true when repeating the experiment (see numbers in the appendix Tab. 7.9).

In summary, of several RNAi inhibitors only CrPV1A revealed efficient RNAi suppression and could be used for further investigations.

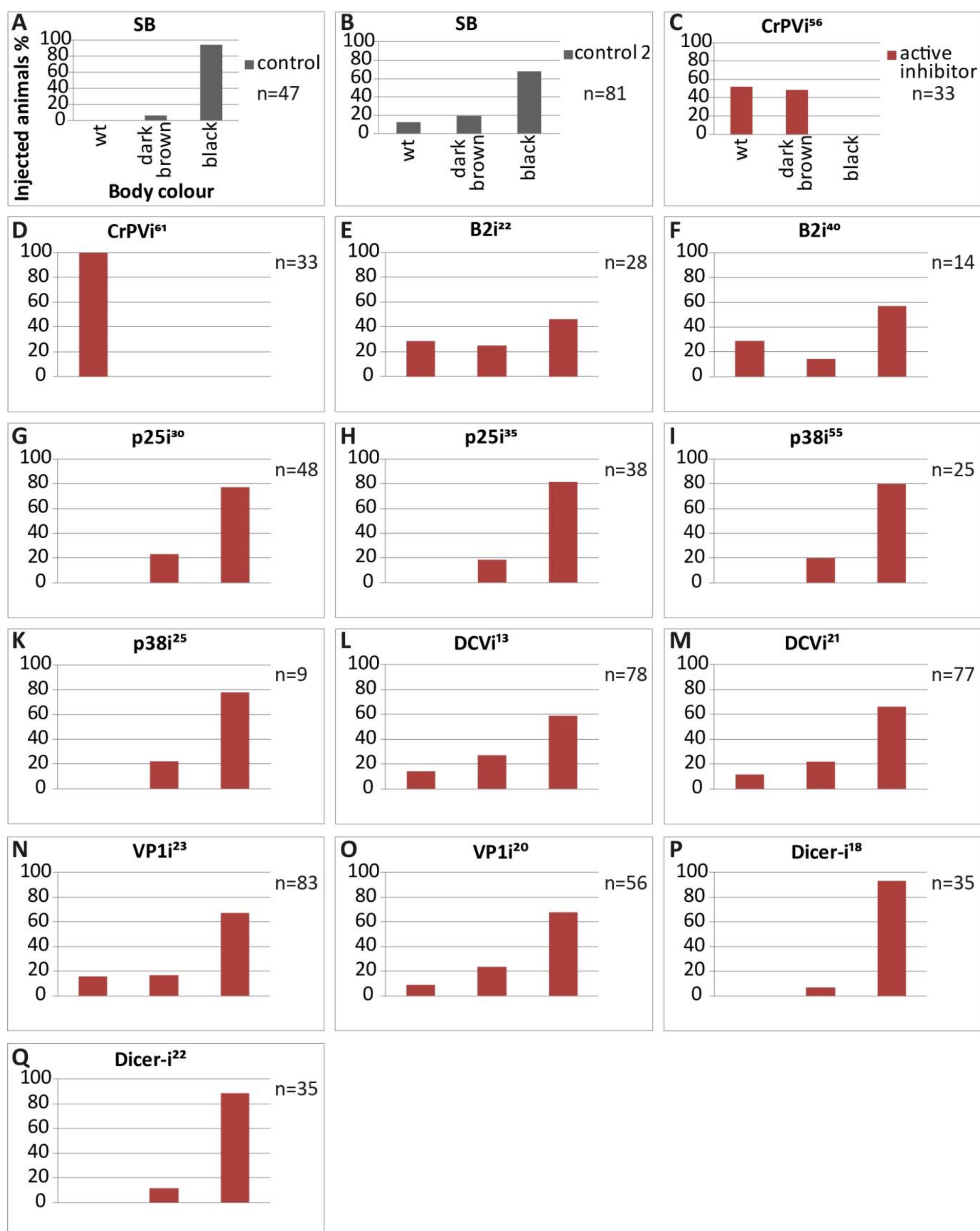


Fig. 4.9: *Tc-ebony* silencing in several inhibitor lines confirmed CrPV1A as an effective RNAi suppressor.

VSR lines expressing different viral proteins (CrPVi, B2i, VP1i, DCVi, p25i, p38i) as well as a *Tc-Dcr-2* IR-line (Dicer-i) were examined for the ability to suppress RNAi by injecting of dsRNA targeting the pigmentation gene *Tc-ebony*. (A-B) Positive controls: Larval RNAi (L6 instar larvae) against *Tc-ebony* (dsRNA concentration: 1.6µg/µl) in the wild type strain, *SB*, led to a black body color in (A) 94% or (B) 68% of all treated animals. (C-O) VSRs were ubiquitously expressed and the respective animals were

injected with *Tc-ebony* dsRNA as the controls. (C-D) Both CrPVi lines displayed efficient RNAi suppression after RNAi. (E-F) FHV B2 expressing strains revealed a diverse body color phenotype after RNAi, indicating a minor RNAi suppression activity. (G-O) The remaining VSR lines did not show obvious RNAi suppression. (P-Q) Larval progeny of Dicer-i lines were treated with *Tc-ebony* dsRNA as described above but no RNAi inhibition was observed.

4.2.5 CrPV1A does not interfere with development and reproduction

For effective, local and/or temporal regulation of the RNAi pathway, the activated viral RNAi suppressor, CrPV1A, should not interfere with viability or development in *Tribolium castaneum*. I tested this by crossing both independent homozygous UAS-CrPVi lines (CrPVi^{56} and CrPVi^{61}) to Boje-Gal4 strain (homozygous) which activated CrPV1A in the offspring. The Boje-Gal4 line appears to express Gal4 ubiquitously in late embryonic, larval, pupal and adult stages but not in early embryonic stages when visualized by crossing to UAS-tGFP. Furthermore, there is no Gal4 expression in the ovaries (data not shown).

As control, UAS-CrPVis, Boje-Gal4 and wild type, *vermillion white*, beetles were outcrossed with wild type beetles and the respective offspring were analyzed like the experimental animals. Each crossing assay was performed with 20 males and 20 females.

First, the developmental velocity of the transgenic progeny in relation to the controls was analyzed. To this end, 0-24h old embryos were collected, counted and incubated at 32°C until larval, pupal and adult stages. Subsequently, it was documented when the first 20 L5-L6 instar larvae, pupae and adults developed (Fig. 4.10 A). The Gal4 driven VSR expression in the progeny (CrPVi^{56} X Boje and CrPVi^{61} X Boje) had no significant effect on the developmental velocity (Fig. 4.10 A). The same was true for the amount of embryos compared to the control crossings (egg numbers ranged from 130 to 151) (see total numbers in grey in Fig. 4.10 A). Further egg collections were analyzed for larval hatch rates (hr), empty eggs (ee) (i.e. eggs which did not develop any cuticle structure) and cuticle phenotypes (i.e. larvae which developed but did not hatch) which were differentiated between cuticles without phenotype (wt) and with cuticle phenotypes (ph) (Fig. 4.10 B). The results revealed similarly high hatching rates ranged from 69-85%, low numbers of empty eggs (14-19%) and very low levels of non-hatched larvae (1-13%), suggesting a quite similar development between the inhibitor strains and the controls. Finally, the comparison

of cuticles of unhatched L1 larvae and their comparison to the controls should indicate whether expression of CrPV1A interferes with development and reproduction. In case of unhatched cuticles, all available L1 larvae were analyzed. In case of hatched cuticles, 25 were scored per collection.

Overall, CrPV1A expressing lines revealed similar defects as the controls (compare C, E with G, I, L, N and D, F with H, K, M, O in Fig. 4.10). The observed nature and frequency of cuticle defects which was also observed in wild types was possibly due to mutations in the genetic background. Importantly, the low numbers of unhatched animals in relation to the high numbers of hatched ones indicated a proper larval development.

In conclusion, expression of the RNAi suppressor, CrPV1A, in late embryonic, larval, pupal and adult stages did not produce significant developmental defects and appeared not to interfere with reproduction. However, due to the lack of a ubiquitous Gal4 driver line, that promotes expression at all developmental stages including early embryos, these findings remain to be confirmed once such a driver is generated.

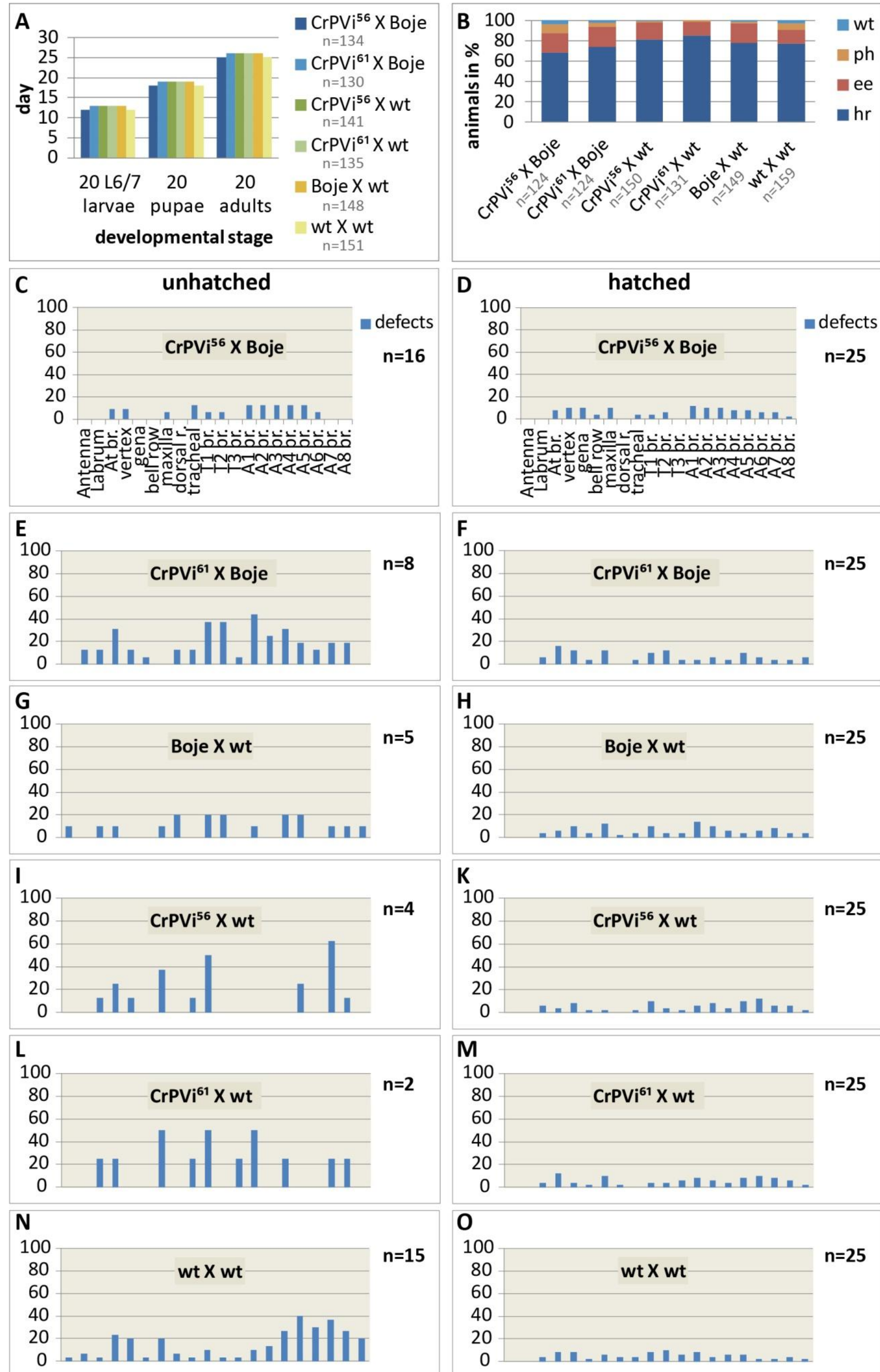


Fig. 4.10: Developmental analysis of CrPV1A expressing animals.

(A) Progeny carrying both, the responder UAS-CrPVi and the driver Boje-Gal4 (both insertion lines: CrPVi^{56} X Boje, CrPVi^{61} X Boje) as well as controls without CrPV1A expression (both insertion lines: CrPVi^{56} X wt, CrPVi^{61} X wt), control with only Gal4 expression (Boje X wt) and wild type control (wt X wt) were analyzed for developmental velocity. Absolute numbers for each egg collection are given in grey. Collected offspring were incubated at 32°C and the day was documented when the first 20 larvae (L5-L6), pupae and adults emerged. (B) Further egg collections of the same crossings were used to investigate the hatch rates (hr), empty eggs (ee) and unhatched larvae that showed a wild type (wt) or a cuticle phenotype (ph) in percent. Absolute numbers for each egg collection are given in grey. (C, E, G, I, L, N) Unhatched cuticles of the same crossings were analyzed for morphological and bristle defects. All available non-hatched larvae were examined. (D, F, H, K, M, O) Hatched larvae of the same crossings were analyzed for cuticle defects. 25 L1 cuticles were scored per hatched collection. Abbreviations for the cuticle defects are given next to the corresponding bar once for each category. At br.: antenna bristles, vertex: vertex triple bristles, gena: gena triplet bristles, bell row: bell row bristles, maxilla: maxilla escort bristles, dorsal r.: dorsal ridge row bristles, tracheal: tracheal openings, T1-A8 br.: affected bristles of the thoracic segments T1-T3 and of the abdominal segments A1-A8.

4.3 Temporal restriction of RNAi

In order to gain temporal control over RNAi, I tested both the triggering of RNAi by the time of injection (called RNAi pulse) and the termination of RNAi by a heat shock mediated expression of the VSR (called hsVSR pulse).

For testing I used the secondary pair-rule gene *Tc-paired* (*Tc-prd*) which is known to be a downstream target of primary pair-rule genes. It is required for the formation of all odd-numbered segments and downregulation of *Tc-prd* produces a typical secondary pair-rule phenotype with some missing gnathal (mandible and labium), thoracic (T2) and odd-numbered abdominal segments (Choe and Brown, 2007; Choe et al., 2006).

4.3.1 Starting of an RNAi pulse by injection in staged embryos failed

At late blastoderm stages the head and thorax anlagen are already established. By embryonic RNAi (eRNAi; injection of dsRNA into embryos) against the gene *Tc-prd* in staged embryos (*SB* strain) I therefore aimed to find convenient developmental time point for the RNAi pulse (eRNAi arrowhead in Fig. 4.11 A) after which gnathal and thoracic segments are built normally, but abdominal segments are affected (Fig.

4.11 A). Upon injection into early elongating embryos, a strong *Tc-prd* phenotype (with a total of 4-5 abdominal segments (AS)) was expected which should decrease with later injections.

Knockdown of *Tc-prd* in staged wild type embryos (aged 5-6h and 6-7h), developed at 32°C, resulted in a phenotype lacking mandible (Md), labium (Lb), second thoracic segment (T2) and urogomphi as well as an abdominal phenotype with a total of 4-5 AS (Fig. 4.11 B upper panels). However, injection into older embryos did not lead to the expected intact gnathal and thoracic segmentation in combination with defective abdominal segments. Indeed, the gnathal and thoracic phenotype seemed to be tightly linked to the abdominal phenotype i.e. both phenotypes were weaker or stronger depending on the timing of injection (see 7-8h, 8-9h, 9-10h old embryos in Fig. 4.11 B upper panels).

In order to check whether these results are due to the rapid development of embryos based on the high incubation temperature (32°C), I decreased the temperature to 25°C. At these conditions, the embryonic incubation time was duplicated because of the slowed down development. It should be noted that for both experiments embryos were incubated at the respective temperature before and after injection until cuticle preparation. Like in the first assay, *Tc-prd* dsRNA injection into 15-16h or 16-17h old embryos led to a strong abdominal phenotype with 4-5 AS accompanied by anterior gnathal and thoracic defects, followed by a mainly wt phenotype when injected into embryos aged 17-18h, 18-19h and 19-20h (Fig. 4.11 B lower panels). Additional tests with younger embryos (10-15 hours) showed the same strong phenotype like described for embryos aged 15-16 hours (data not shown). Hence, *Tc-prd* RNAi in embryos with slowed embryonic development did not result in the expected normal gnathal and thoracic development but defective abdominal segmentation.

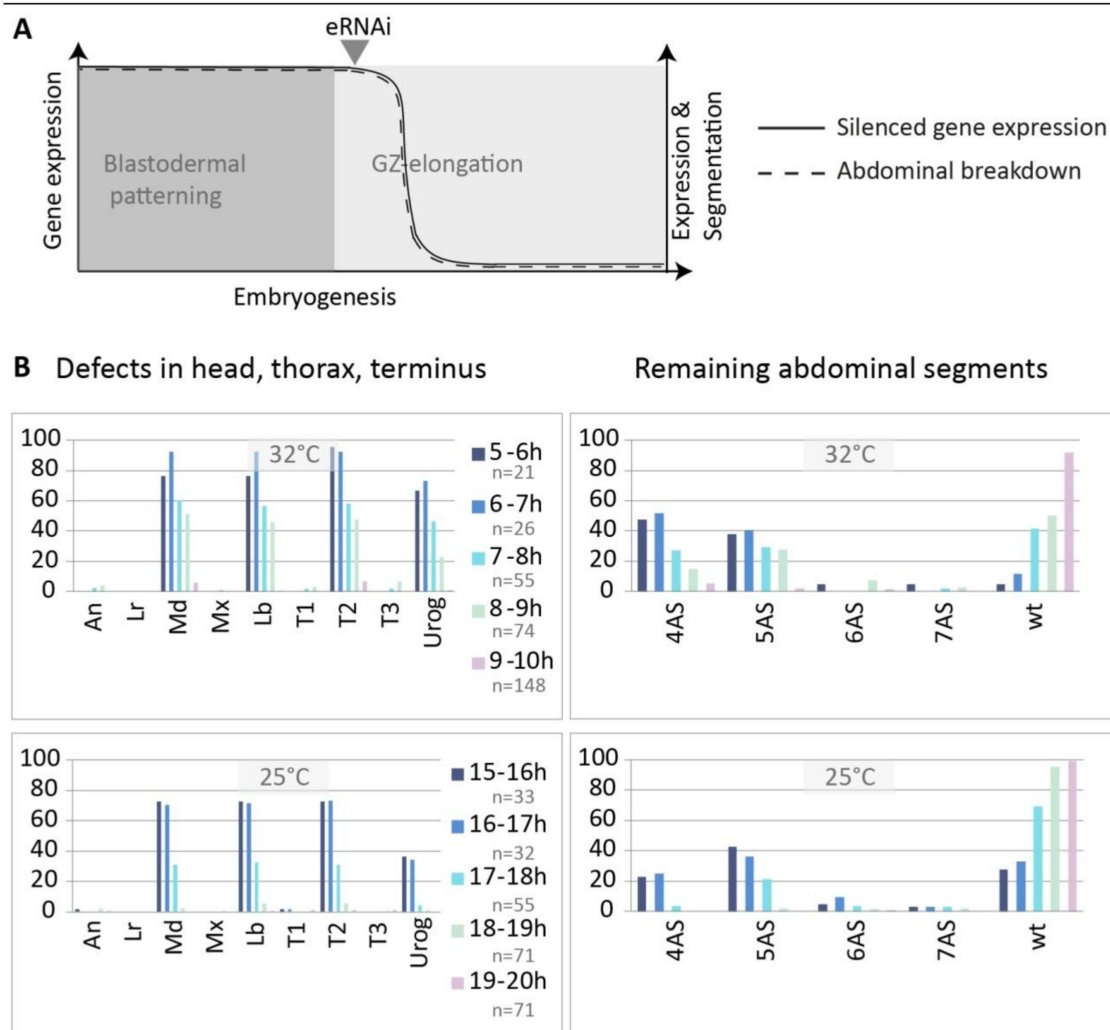


Fig. 4.11: Induction of RNAi by injection.

(A) Scheme for the starting of RNAi by embryonic injection. Upon injection of dsRNA (eRNAi arrowhead) into staged embryos the expression of *Tc-prd* is expected to decrease (solid line). As blastodermal phase is unaffected by *Tc-prd* RNAi, only abdominal segments should be impaired (dashed line).

(B) dsRNA of the secondary pair-rule gene *Tc-prd* was injected into staged embryos and cuticles were analyzed for anterior defects as well as the number of remaining abdominal segments. The absolute numbers for each cuticle analysis are given in grey. Before the injection procedure, embryos were kept at 32°C (upper panels) or 25°C (lower panels) until the respective age (5-6h to 9-10h and 15-16h to 19-20h) and after treatment, incubated at the same temperature for four or eight days, respectively, until cuticle preparations. The amount of cuticle defects of the head, thorax and urogomphi are shown in the left panels. The numbers of the remaining abdominal segments are shown in the panels on the right side. Abbreviations for the cuticle defects are given next to the corresponding bar. An: antenna, Lr: labrum, Md: mandible, Mx: maxilla, Lb: labium, T1-T3: thoracic segments 1 to 3, Urog: urogomphi, 4AS-7AS: a total of four to seven abdominal segments, wt: wild type phenotype.

4.3.2 Heat shock induced VSR expression rescues abdominal segmentation of *Tc-prd* RNAi embryos

Next, I asked whether it was possible to suppress the RNAi effect by activation of VSR during GZ-elongation in order to regain previously silenced gene expression. For that purpose, *Tc-prd* served again as control gene. Two transgenic hsVSR lines (hsCrPVi^{47} and hsCrPVi^{49}) were generated, carrying CrPV1A under the control of a heat shock promoter (Schinko et al., 2012) (see materials and methods). Both insertion lines were tested in the rescue experiments. Heterozygous or homozygous hsVSR pupae were selected for their DsRed eye marker. Female pupae of these animals were injected with *Tc-prd* dsRNA (2.7µg/µl) and, subsequently, crossed to transgenic males for mating and egg deposition. Hence, at least 75% of the embryonic offspring should be heterozygous or homozygous for the transgene hsCrPVi. Parental RNAi against *Tc-prd* (pRNAi arrowhead in Fig. 4.12) should affect embryonic blastodermal patterning, leading to anterior structure defects in the gnathal and thoracic segments of the progeny. The subsequent heat shock mediated VSR activation (hsVSR in Fig. 4.12) during elongation should be able to recover the expression of the secondary pair-rule gene *Tc-prd,* resulting in a rescued abdominal segmentation (Fig. 4.12 increasing lines). The segmentation was considered to be rescued if the heat shocked *Tc-prd* RNAi cuticles revealed six to eight AS in contrast to the non-heat shocked control *Tc-prd* RNAis, which showed a strong abdominal phenotype with four to five AS (Fig. 4.13, Fig. 4.14 B). In order to establish the proper time point for the VSR pulse, RNAi embryos developed at 32°C until they reached germ rudiment to elongation stages (egg collections of 10-15h, 11-16h and 12-17h old embryos) and then heat shocked for 10 minutes at 48°C to activate the RNAi suppressor (see materials and methods) (Fig. 4.12, 4.13). In parallel, wt embryos of the same age were stained for *Tc-wg* expression to determine the embryonic stage at the heat shock treatment (Fig. 4.12). Furthermore, transgenic, non-heat shocked *Tc-prd* RNAi embryos and non-injected but heat shocked embryos were used as controls (Fig. 4.13).

As expected, VSR activation in staged *Tc-prd* RNAi embryos of the lines hsCrPVi^{47} and hsCrPVi^{49} resulted in a strong defective gnathal and thoracic phenotype (Fig. 4.13 A-B left panels, Fig. 4.14 B-E) but rescued abdominal phenotype (Fig. 4.13 A-B right panels, Fig. 4.14 D). This phenotype was observed in each egg collection (Fig. 4.13). Especially, heat shocks of transgenic RNAi embryos

at the age of 10-15h led to a high number of cuticles with restored abdomen (38% of line hsCrPVi[47] and 25% of hsCrPVi[49]) (Fig. 4.13). The remaining treated embryos revealed either a shortened abdomen with 4-5 AS (Fig. 4.13, Fig. 4.14 C) or undefined segmentation number (called "no segmentation" in Fig. 4.13 right panels, Fig. 4.14 E).

Furthermore, the transgenic line hsCrPVi[49] tended to exhibit lower cuticle numbers with rescued AS than line hsCrPVi[47], indicating some position effect (Fig. 4.13, compare right panels of A and B).

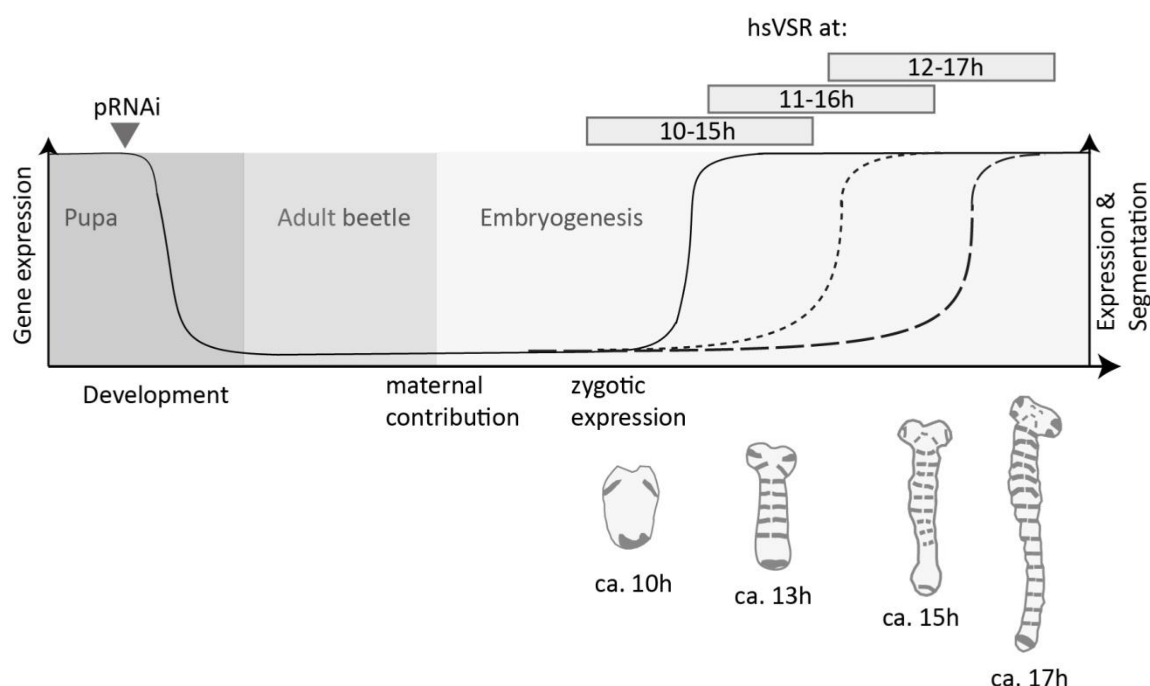


Fig. 4.12: Model for the abdominal rescue approach by VSR activation.
Parental RNAi (arrowhead) of the secondary pair-rule gene *Tc-prd* silenced the expression (decreasing solid line represents the downregulated gene expression) in pupal, adult and embryonic stages. In order to rescue *Tc-prd* function during GZ-elongation, egg collections with embryos aged 10-15h, 11-16h and 12-17h (developed at 32°C) were heat shocked twice for 10 minutes at 48°C (see further details in materials and methods) to start VSR expression (bars indicate the age of the respective egg collections at the time when the first heat shock was performed). Suppressor activation in staged embryos resulted in rescued *Tc-prd* expression, associated with rescued abdominal segmentation (increasing lines represent rescued gene expression and rescued segmentation; solid line for 10-15h old embryos, dotted line for 11-16h old embryos and dashed line for 12-17h old embryos). Anterior segmentation was, however, impaired due to the knockdown of *Tc-prd* expression (decreased line during embryogenesis). Embryonic developmental stages corresponding to the timing of the first heat shock were determined by *Tc-wg* staining. As an example, a 10h old germ rudiment, a

13h old germ band with five *Tc-wg* stripes, a 15h old elongating germ band with nine *Tc-wg* stripes and a fully elongated 17h old germ band stage with fifteen *Tc-wg* stripes are shown (anterior is up).

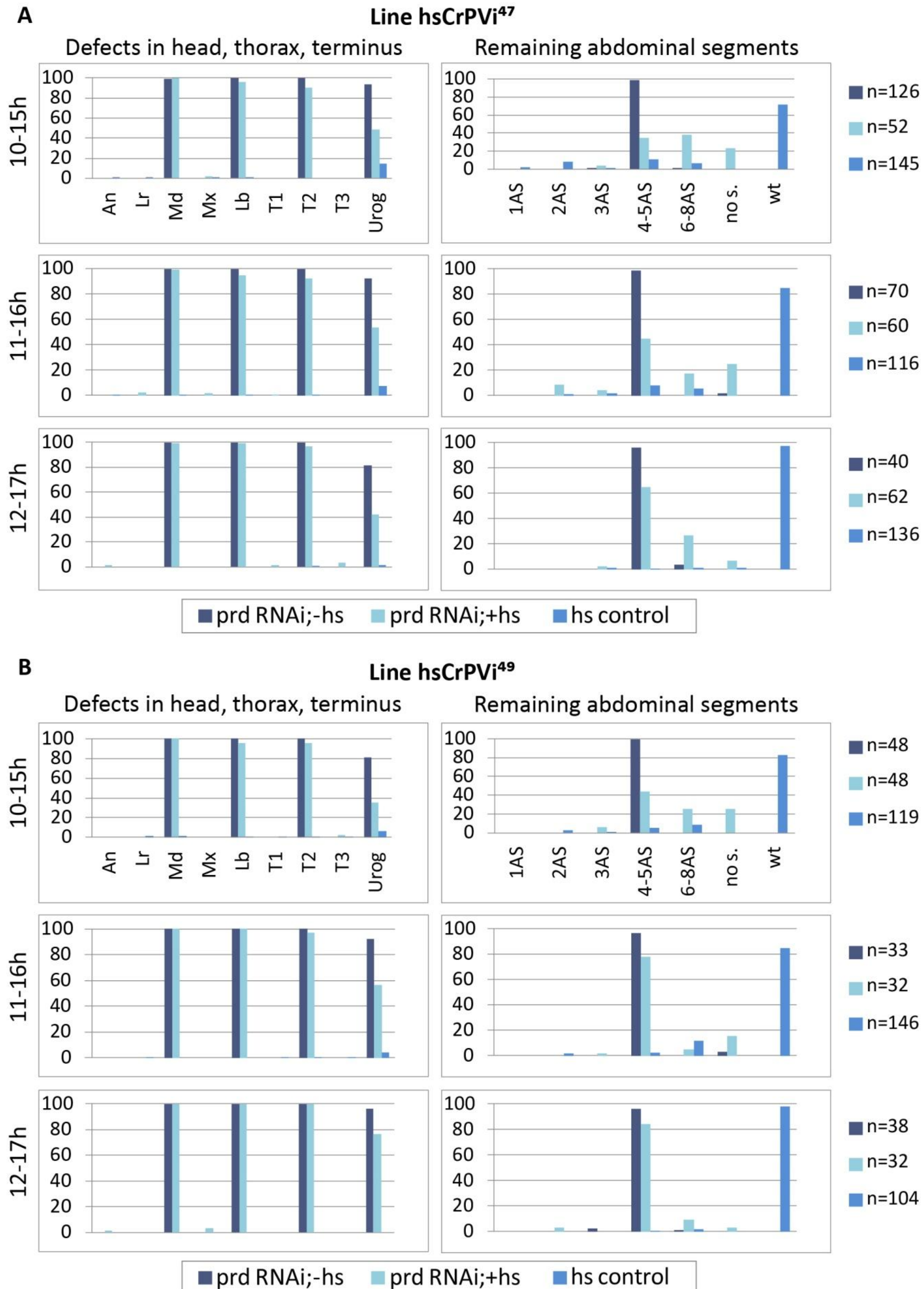


Fig. 4.13: Cuticle defects post parental *Tc-prd* RNAi followed by RNAi rescue in staged embryos

(A, B)Two transgenic insertion lines carrying heat shock inducible CrPV1A were used (hsCrPVi[47] in A and hsCrPVi[49] in B) for the abdominal rescue approach. pRNAi against *Tc-prd* was followed by heat shock mediated CrPV1A activation in staged embryos (prd RNAi;+hs) (see respective age at the left). Absolute numbers for each cuticle analysis are given at the right. The percentage of cuticle defects in head, thorax and urogomphi are shown in the left panels. The numbers of remaining abdominal segments are shown in the right panels. Transgenic, non-heat shocked *Tc-prd* RNAi embryos (prd RNAi; -hs) served as RNAi controls. Transgenic, non-injected but heat shocked embryos (hs control) served as heat shock controls. Abbreviations for the cuticle defects are given next to the corresponding bar once for each insertion line and category. An: antenna, Lr: labrum, Md: mandible, Mx: maxilla, Lb: labium, T1-T3: thoracic segments 1 to 3, Urog: urogomphi, 1AS-8AS: a total of one to eight abdominal segments, no s.: no visible abdominal segments, wt: wild type phenotype.

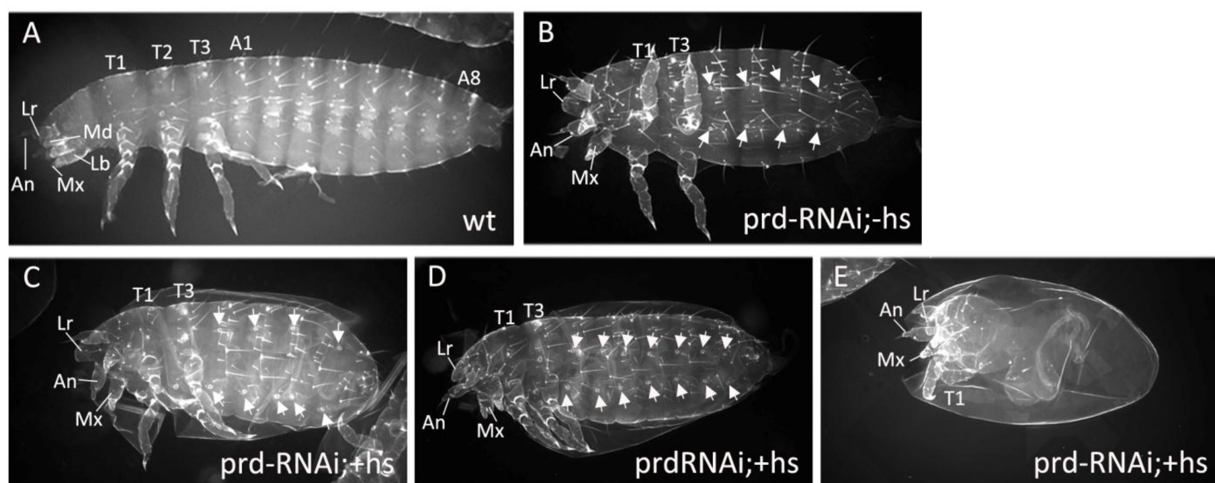


Fig. 4.14: L1 larval cuticle phenotype classes after *Tc-prd* pRNAi and RNAi rescue by VSR activation.

Anterior is to the left. (A-E) Lateral views. (B-E) Transgenic embryos carried hsCrPV1A (at least 75%). (A) Wild type untreated L1 cuticle. (B) Non-heat shocked *Tc-prd* RNAi control cuticle represents a strong RNAi phenotype with missing gnathal segments (mandibles and labium) and thoracic segment T2 as well as with remaining 4 AS (white arrows). (C) Heat shocked *Tc-prd* RNAi cuticle with 4-5 AS (white arrows) and gnathal and thoracic defects like in B, indicating that the rescue was not successful either due to the age of the embryo or because the embryo did not inherit the construct (25%). (D) Heat shocked *Tc-prd* RNAi cuticle with 7 AS (white arrows) and gnathal and thorax defects like in B, indicating that the RNAi effect was successfully rescued. (E) Heat shocked *Tc-prd* RNAi cuticle represents the “no segmentation” phenotype. Cuticle shows gnathal defects like in B and missing thoracic (T2 and T3) and abdominal segments. Abbreviations: An: antenna, Lr: labrum, Md: mandible, Mx: maxilla, Lb: labium, T1-T3: thoracic segments 1 to 3, A1-A8: abdominal segments 1 to 8, wt: wild type.

5 Discussion

5.1 RNAi target genes for pest control

5.1.1 Necessity for more efficient target genes

RNA interference appears to be a promising method for pest control. Sequence-specific gene silencing by RNAi enables targeting of individual or closely related species selectively. The fact that some insects are able to trigger RNAi response after dsRNA ingestion led to the development of insecticidal RNAi approaches in plants (Baum et al., 2007; Mao et al., 2007). For instance, transgenic corn plants have been engineered to express dsRNA targeting the *vacuolar ATPase* gene of the western corn rootworm. Upon feeding on these plants, the dsRNA induced gene silencing in the WCR, resulting in its death (Baum et al., 2007). Despite this efficiency, only early larval stages of the western corn rootworm were affected by ingestion of plant derived dsRNA, indicating that the effectiveness of target gene silencing needs to be increased (Bucher, personal communication).

Potential reasons for this observation might be limited or insufficient dsRNA expression levels in transgenic plants to elicit an RNAi response in late larval stages of WCR. It is suggested that high doses of dsRNAs are required to trigger a strong RNAi effect upon ingestion (Terenius et al., 2011; Tian et al., 2009; Zhang et al., 2013). Besides, it has been demonstrated that the production of dsRNAs in transgenic plants is limited because it is hampered by endogenous plant RNAi machinery, which cleaves long dsRNAs into siRNAs (Pitino et al., 2011; Thakur et al., 2014; Zha et al., 2011). These siRNAs are less potent than long dsRNAs possibly because short dsRNAs (≤60 bp) are not efficiently taken up by insect cells (Kumar et al., 2012; Mao et al., 2007; Miller et al., 2012).

The identification of efficient target genes that kill the pest even at low dsRNA concentrations independent of the developmental stage of the organism is thus essential for RNAi based pest control techniques. Unfortunately, the detection of suitable RNAi target genes by high-throughput-screens is not feasible in pest species due to missing genomic and genetic tools and difficult rearing conditions.

5.1.2 The large scale RNAi screen reveals novel genes for application in pest control

The large scale, unbiased RNAi screen, *iBeetle*, in the red flour beetle, *Tribolium castaneum* is a great opportunity to identify novel gene functions which might be more representative for insects because the beetle development is believed to be more ancestral than that of *Drosophila* (Bucher and Wimmer, 2005; Klingler, 2004; Lynch and Roth, 2011; Schröder et al., 2008; Tautz et al., 1994). In the *iBeetle* screen, a large number of genes were investigated, resulting in the identification of novel genes that induced lethality upon knockdown. (Schmitt-Engel et al., accepted) (Dönitz et al., 2015). Indeed, by re-injection and titration experiments, I selected the best 40 and eleven RNAi target genes which caused organism death even when provided in minute amounts. This high mortality rate is an important factor for the application of target genes in RNAi-mediated crop protection as already mentioned in section 5.1.

The most efficient eleven RNAi target genes induced lethality at different developmental stages in *Tribolium* (Fig. 4.1, Fig. 4.2) and did not show any strain specificity since they were tested in three different strains: the *pig19* and *D17Xred* strains during the *iBeetle* screen and the *San Bernardino* strain during my thesis (Schmitt-Engel et al., accepted) (Kitzmann et al., 2013). The efficacy of these genes is therefore based on their essential roles during development, probably due to housekeeping functions. Hence, the respective orthologs are likely to be effective targets in other pest species.

5.1.2.1 Tests in further pest species are required

The analysis of the eleven RNAi target genes revealed a more efficient RNAi effect and therewith a much higher mortality rate than the orthologs of previously published genes (Baum et al., 2007) which I tested in *Tribolium* by dsRNA injection (Fig. 4.1 N-R). However, these genes induced lethality in WCR upon dsRNA ingestion, while all RNAi experiments in *Tribolium* were performed by injection procedure. Moreover, it has been shown that the effectiveness of a target gene might vary among species which could be due to varied susceptibility of several organisms to different RNAi targets (Kwon et al., 2013; Li et al., 2013, 2011a, 2011b; Upadhyay et al., 2011). To investigate the efficacy of the identified RNAi target genes, dsRNA

feeding approaches in further pest species are therefore necessary. Although high-throughput screening of thousands of genes is challenged in pest species, the testing of a restricted set of RNAi target genes (namely ten to 40 targets) is feasible.

5.1.2.2 Off target effects on non-target organisms cannot be excluded but are improbable

The high selectivity of RNAi-mediated crop protection is based on the nucleotide sequence identity of the dsRNA to its target gene sequence. However, downregulation of genes in non-target organisms have also been reported (Baum et al., 2007). For instance, dsRNA against *vATPase* of the Colorado potato beetle (CPB; *Leptinotarsa decemlineata*) also silenced the orthologous gene in the western corn rootworm, indicating an off target effect on a non-target organism (Baum et al., 2007). Hence, to reduce off target effects in non-target organisms it has been suggested to use RNAi target genes which are species-specific. The analysis of the eleven *Tribolium* RNAi targets, however, indicated that species-specific genes will have as many off targets in non-target organisms as highly conserved proteins. This became apparent when potential off target sites, targeting genes of other species, were plotted against their RNAi target gene nucleotide sequence. There was no obvious correlation between the number or location of potential off targets and conserved protein regions (see black bars in Fig. 4.4). In contrast, off target stretches were also found in less conserved non-coding UTRs. This indicates unpredictable, evenly distributed putative off target sites (see open bars in Fig. 4.4). Additionally, a *Tribolium*-specific gene also led to putative off target sites in a non-target organism as demonstrated in *Aedes aegypti.* Although *Aedes aegypti* missed an orthologous gene of L76 (*hsc70-3*) (Fig. 4.6 A; Fig. 7.2 H), the search for off targets in the transcriptome database of this species resulted in potential off target stretches when using the *Tribolium* specific L76 nucleotide sequence (Fig. 4.4 H).

Moreover, the more species were added to this analysis, the more difficult it was to find off target free stretches. It should be noted, that potential off target sites were identified by searching the nucleotide sequences for siRNAs with an exact sequence identity of ≥17 nucleotides (Kulkarni et al., 2006). However, further reports have shown that siRNAs with as few as seven nucleotides of sequence complementarity to an mRNA can also trigger silencing of unintended genes (Birmingham et al., 2006; Jackson et al., 2006; Lin et al., 2005). These results indicate that it is difficult if not

impossible to predict dsRNA fragments without off target sites in non-target organisms even when using species-specific genes.

Despite this finding, the species specificity of the RNAi based pest control technique remains unchallenged when compared to other methods that usually target all or at least many species. The chance that in silico predicted siRNAs of an arbitrary range of length (from 17 to 29 nt) will induce off target effects range from 5 to 80% (Qiu et al., 2005). It is therefore impossible to predict whether the identified potential off target sites will induce an RNAi response in non-target organisms. However, at least half of the unwantedly target genes will not result in lethality due to the fact that about 50% of investigated genes in large scale screens lead to a phenotype when mutated or silenced in *Drosophila* or *Tribolium* (Schmitt-Engel et al., accepted) (Mullins et al., 1994; Nusslein-Volhard, 1994; Wieschaus et al., 1984). Further, only individuals that eat the protected plants will suffer. And finally, only a small number of non-target organisms will be able to induce RNAi response after dsRNA digestion because many species lack a dsRNA uptake mechanism in gut cells (Huvenne and Smagghe, 2010).

These results indicate that efforts to increase safety should focus on selected species (e.g. beneficial insects, other herbivores) that need to be protected in the respective given ecological setting. This can be accomplished by designing target sequences without potential off target stretches in these organisms. Furthermore, selected dsRNAs could be tested in vivo by feeding treatments in the respective species.

5.1.3 GO term clusters are predictive for efficient RNAi target genes and identify the proteasome as prime target

By using the top 40 RNAi target genes, GO term clusters were identified that are predictive for genes with lethal phenotype upon RNAi. The search for the most efficient RNAi targets in e.g. gut transcriptomes of pest species may therefore be accelerated by selecting genes that show these GO term combinations.

Furthermore, it was possible to detect the proteasome as prime RNAi target by using these clusters. The first two clusters revealed the highest biological significance (due to the highest enrichment score; see materials and methods) and were related to proteasome function (Fig. 4.6 C). The significance of proteasome

function was confirmed by the fact that almost all novel *Tribolium* orthologs that shared the same GO term annotations and were analyzed in the *iBeetle* screen showed a strong lethal phenotype upon RNAi (Fig. 4.6 B).

The proteasome is a multi-protein complex which is composed of at least 32 different proteins. It is conserved among eukaryotic cells and plays an important role in catalyzing various essential functions (see section 2.1.4). Therefore, silencing of genes involved in proteasome assembly or in related pathways might be an excellent method for RNAi-mediated pest control. For instance, it has been demonstrated that downregulation of a proteasome subunit gene, *Rpn7*, in the root knot nematode, *Meloidogyne incognita*, led to a significant reduction of nematode motility and reproduction. The same was observed when the nematode fed on transgenic plants which were engineered to express dsRNA against *Rpn7*, indicating that proteasome genes are promising candidates for controlling this plant parasitic nematode (Niu et al., 2012). Further studies concerning the 26S proteasome function in *C. elegans* have shown that RNAis targeting different 26S proteasome subunit genes resulted in embryonic and postembryonic lethality, indicating an essential function of these genes (Takahashi et al., 2002). Moreover, two of the most efficient eleven RNAi target genes in this thesis are subunits of the 26S proteasome (L80 and L84, see Fig. 4.6 A) and seven proteasomal genes rank among the best 40 RNAi targets (see Fig. 4.6 C and appendix Tab. 7.5). In total, fourteen different genes (seven genes of the top 40 targets and seven novel genes in the GO term analysis; see cluster 1 in the appendix Tab. 7.6) involved in proteasome assembly or pathway were recognized in this study. Of these, thirteen induced significant mortality when downregulated in *Tribolium*. It will be interesting to test whether further genes of the proteasome pathway will elicit the same strong lethality in RNAi approaches. Nevertheless, these results coincide with the assumption that genes related to proteasome function might be ideal target genes for pest control. However, the proteasome pathway is conserved across different species. Therefore, biosafety is only achieved by RNAi mediated approaches due to the fact that the pathway can be triggered in a sequence-specific way. The application of chemicals against this pathway would affect non-target organisms.

5.1.3.1 Potential reasons for the failure to identify predictive GO term clusters for the top eleven RNAi target genes

It was not possible to identify predictive GO term combinations for the most effective eleven RNAi target genes, indicating that these genes either do not share similarities or existing similarities were not detected. As not all genes are linked to a GO term annotation, clustering was incomplete and might lack important term combinations. Furthermore, functional annotation clustering of GO terms for the eleven RNAi target genes resulted in three clusters (data not shown) but it was not possible to find *Drosophila* genes that shared the respective annotations. A possible explanation is that GO terms change with new discoveries, resulting in redundant or obsolete annotations. In line with this assumption, the last DAVID database update was in 2009 (Huang et al., 2009a, 2009b) whereas the Flybase Query Builder, which was used to find *Drosophila* genes that shared the respective GO term combinations, was last updated in 2013 (Gelbart et al., 1997). Some GO annotations might therefore be not compatible with the Flybase database. The same “not up-to-date situation” was observed when using other tools for GO term analysis like e.g. WebGestalt at that time (data not shown) (Zhang et al., 2005). Finally, an input number of eleven genes might be insufficient to perform statistical analyses, leading to the identification of rather general terms. Thus, large gene lists have higher statistical power, resulting in higher sensitivity for more specific terms.

5.1.4 Possibilities to increase the efficacy of the identified RNAi target genes

As already mentioned, if RNAi target genes are expressed in transgenic plants, their efficiency can be reduced by endogenous plant RNAi machinery (Pitino et al., 2011; Thakur et al., 2014; Zha et al., 2011). It has been suggested that combinatorial RNAi could enhance the effectiveness of RNAi based control strategies by e.g. synergistic effects (Gu and Knipple, 2013; Price and Gatehouse, 2008). However, my results demonstrate that silencing of different RNAi target genes in double knockdowns do not result in synergism and increased lethality of the organisms (Fig. 4.3).This indicates that either more genes should be tested for synergism or that the efficiency of RNAi mediated pest control cannot be enhanced by synergistic action in combinatorial RNAis. Therefore, further methods are required to improve the pest control approach.

One possibility to overcome the reduction of RNAi effects could be a target gene expression in plant chloroplasts. It has been shown that chloroplasts lack an endogenous RNAi machinery. Therefore, the expression of dsRNAs in these cellular compartments of transgenic tomato plants led to stable dsRNA production (Zhang et al., 2015). Chloroplast expression of long dsRNAs should thus be the preferred way to retain the effectiveness of identified RNAi target genes.

5.1.5 The identification of more efficient RNAi target genes is unlikely

The most efficient RNAi target genes induced a mortality rate of 100% within eight days after injection even when dsRNAs were provided in minute amounts (Fig. 4.1). As the *iBeetle* screen comprised one third of the genome, it is not likely that any gene will be found which induces organism death more rapidly. However, it remains unclear why there is a time-lag between the RNAi treatment and the organism death. Such a time delay has already been reported for WCR as well as the flea beetle (Baum et al., 2007; Zhao et al., 2008). On the one hand, RNAi is not able to affect already produced proteins. Hence, it takes time until the respective proteins are degraded, resulting in a time-lagged organism mortality. On the other hand, it is unknown how fast the dsRNA is distributed and enters the cells. Quantitative real time PCR (RT-qPCR) would be helpful to investigate this assumed time-lag in *Tribolium.* Finally, the requirement for RNAi amplification could account for the delayed mortality. Although insects apparently lack an RdRP which is responsible for RNAi amplification and systemic spread in *C. elegans*, RNAi amplification in *Tribolium* might be based on a different mechanism (Sijen et al., 2001; Tomoyasu et al., 2008). Such an amplification mechanism could explain the fact that low concentrated dsRNAs triggered organism death but with delay.

5.2 Investigation of RNAi suppressors in *Tribolium*

5.2.1 The viral protein CrPV1A reveals a broad RNAi suppression activity among different species including *Tribolium*

A method by which the RNAi mechanism can be suppressed might allow controlling the pathway in different ways. For instance, by temporal repression and activation of the RNAi mechanism, the time point when gene silencing should occur can be determined. Local inhibition of RNAi might permit gene silencing in restricted tissues.

To identify an effective way for RNAi inhibition, six different viral suppressors of RNAi as well as transgenic RNAi, targeting the *Dicer-2* gene, were tested. Of these, the *Cricket Paralysis virus* protein CrPV1A was the most potent suppressor of RNAi in *Tribolium* (Fig. 4.8, Fig. 4.9). These results correspond to studies in which the *Cricket Paralysis virus* has been reported to be a highly efficient virus of several species in the lab, indicating a broad activity among different organisms (Nayak et al., 2010; Plus et al., 1978; Reinganum et al., 1970). This broad host spectrum may rely on a broadly effective RNAi inhibition mechanism.

5.2.1.1 Failed test of local suppression of CrPV1A: Experimental design or limitation of the technique?

Spatial RNAi inhibition is an important factor when establishing a tool for gene functional studies in specific tissues. In order to investigate whether CrPV1A is able to locally suppress RNAi, EGFP was expressed ubiquitously (by heat shock activation) while the VSR was activated in a nested domain (by Gal4/UAS). After silencing, EGFP should be suppressed in all tissues except for those that expressed an active VSR. However, it was not possible to confirm that CrPV1A locally inhibits the RNAi pathway. Likewise, it was not possible to locally suppress the silencing of heat shock activated ubiquitous EGFP expression (Fig. 4.7).

Potential ubiquitous CrPV1A activity

An important factor for this analysis was a continuous local expression of RNAi inhibitor. In my approach the VSR was activated by the BB-Gal4 driver line which

displayed spatially restricted expression pattern in late larval, pupal and adult stages but ubiquitous expression in early larval stages. Hence, the activation of the VSR by BB-Gal4 in young larvae resulted in initially ubiquitous suppressor protein activity, which might still operate (depending on the protein stability) when Gal4 expression was already restricted to the BB pattern. Upon EGFP RNAi, the silencing of EGFP might therefore be not only prevented in the BB region but rather in the entire animal.

Local suppression might be impeded by spread of CrPV1A throughout the body

Several reports in plants have shown that plant viruses defective for their RNAi-suppressors displayed an impaired long distance movement and were even incapable to move from cell-to-cell (Chu et al., 2000; Hacker et al., 1992; Kasschau and Carrington, 2001). Further studies have investigated the situation in fruit flies which were injected with a recombinant *Sindbis virus* that was engineered to express GFP and the RNAi suppressor CrPV1A. *Sindbis virus* that expressed CrPV1A showed a dramatic increase of virus replication (visualized by GFP), suggesting that the RNAi suppressor might be involved in virus spread (Nayak et al., 2010). This could be due to a potential capability of CrPV1A to cross cell boundaries. If this is the case, then locally expressed VSR protein might spread from the BB-restricted tissue (where it was activated by Gal4/UAS) throughout the whole organism, resulting in widespread suppression of the RNAi mechanism. This might also explain why downregulation of the ubiquitously expressed EGFP did not result in the expected BB-specific EGFP fluorescence (Fig. 4.7).

Alternatively, the reported virus replication and spreading might be just a consequence of VSR-mediated RNAi suppression but not VSR spreading itself. The RNAi machinery functions as an antiviral defense response to limit the extent of virus invasion. This limitation is mostly accompanied by systemic spreading of a virus-specific immunity signal (i.e. siRNAs) to uninfected tissues (Saleh et al., 2009; Voinnet, 2005). Most RNAi suppressors interfere with this signal and might thereby enable viral movement and infection of surrounding cells (Lakatos et al., 2006; Li et al., 2002; Lingel and Sattler, 2005; Scholthof, 2006; Voinnet et al., 1999). Therefore, the question whether CrPV1A is actually capable to spread from its expression location remains unclear and needs further investigation. Western blot analyses

could quantify inhibitor protein in various tissues (e.g. BB tissue versus abdomen) and thereby clarify whether VSR can spread in *Tribolium*.

Improvements in the experimental design are necessary

The argumentations about locally non-restricted inhibitor activity do not explain why no differences in EGFP fluorescence signal in relation to the controls were recognized. For instance, if CrPV1A was indeed active in the entire animals and thereby inhibited EGFP downregulation in the pupae, there should be obvious differences in fluorescence between the pupae of the treatment and the control. But it was not the case. In all pupae, EGFP expression appeared to be downregulated to the same extent. Hence, it could be that the heat shock mediated EGFP expression level was not high in the BB tissue. It is not known, what tissue or organ is marked by BB and it is not certain whether EGFP was efficiently activated in the VSR expressing tissue by the heat shock. In that case, CrPV1A was probably able to rescue EGFP signal in the BB domain, but this signal might have been too faint to be apparent. Further, a faint BB-EGFP fluorescence might also be masked by the autofluorescence of pupal wings. Moreover, EGFP expression was activated by performing one heat shock pulse in *Tribolium* pupae. However, there is some evidence that two heat shocks with a regeneration time of two hours in between might be more efficient in activating gene expression (Oberhofer, 2014). Further tests with two heat shocks for target gene expression could clarify this issue. Besides, different driver, reporter and target gene strains should be used to test the ability of this VSR to locally inhibit the RNAi mechanism.

A line with *Tc-polyubiquitin* driven GFP expression might reveal a much stronger signal than the heat shock line (Lorenzen et al., 2002b). Another possibility would be the activation of the VSR in *Tribolium* wings by a Gal4 driver line which has recently been identified during an enhancer trap screen in the lab (Bucher, personal communication). Silencing of the pigmentation gene *Tc-ebony* in this genetic background could clarify the question about the potential of the RNAi inhibitor to locally suppress RNAi. An adult phenotype with wild type, red-brown wings but black body color phenotype would indicate a disrupted RNAi mechanism exclusive in the wings but intact mechanism in the remaining tissues.

5.2.1.2 CrPV1A has probably no effect on the microRNA pathway

The identification of the viral RNAi suppressor CrPV1A which efficiently impaired the RNAi mechanism in *Tribolium* raised the question of whether this inhibitor will interfere with the viability or development when ubiquitously expressed in transgenic organisms. This is important for further application of CrPV1A to regulate gene silencing in temporal and/or spatial manner. Developmental defects would hamper gene functional studies, leading to invalid results. In *Drosophila*, for example, CrPV1A neither affects the normal physiology nor the development of transgenic flies (Nayak et al., 2010). The same could be true for *Tribolium* based on my experiments. Except for some bristle defects, ubiquitously activated CrPV1A expression did not significantly influence the viability or development of transgenic progeny compared to the controls (Fig. 4.10). Due to similar frequency of affected bristles in the controls, these defects might be explained by mutations in the genetic background.

Furthermore, CrPV1A expression in transgenic fruit flies does not interfere with the miRNA pathway which plays an important role in different developmental processes (Nayak et al., 2010). Viral impairment of miRNAs is mostly accompanied by developmental defects in the organisms which resemble defects observed in mutant species deficient in their miRNA pathway (Chapman et al., 2004; Dunoyer et al., 2004; Lecellier et al., 2005; Pasquinelli et al., 2005; Yu et al., 2006). The fact that CrPV1A did not significantly affect *Tribolium* development might indicate an intact, unaffected miRNA pathway.

However, CrPV1A was activated by a Gal4 driver line which expressed the inhibitor in late embryonic, larval, pupal and adult stages but not in early embryos. Hence, some interference with viability might have been missed. A transgenic line which ubiquitously and constitutively expresses CrPV1A in all developmental stages (driven by e.g. *polyubiquitin* promoter) is necessary to confirm my results.

5.2.2 VP1 and CrPV1A reveal similar suppression mechanism but different potency

The *Nora virus* suppressor protein VP1 acts like CrPV1A by Ago-2 inhibition (van Mierlo et al., 2012; Nayak et al., 2010). Despite the similar mechanism, both proteins revealed different RNAi suppression outcomes. While CrPV1A efficiently interfered with RNAi in *Tribolium*, there was no obvious suppression activity of VP1 (Fig. 4.7,

4.8, 4.9). Presumably both inhibitor proteins might interact with different domains or sequences of Ago-2. For instance, CrPV1A might bind conserved domains of Ago-2, like PIWI or PAZ, while VP1 might act on species-specific non-conserved sequences (van Mierlo et al., 2014; Obbard et al., 2006). This assumption corresponds to recently published data in which suppressor proteins of several Nora-like viruses of diverse *Drosophila* species have been demonstrated to be highly host-specific (van Mierlo et al., 2014). Therefore, despite the same inhibition mode of CrPV1A and VP1 the exact interaction domains or sequences might be of prime importance for efficient RNAi silencing in different species.

5.2.3 Potential reasons for the failure of FHV B2 and DCV1A

The FHV B2 is able to inhibit the RNAi mechanism in several organisms like *Drosophila*, *C. elegans,* as well as plants (Li et al., 2002; Lingel et al., 2005; Lu et al., 2005; Singh et al., 2009). The mode of action of FHV B2 relies on its ability to bind dsRNAs as well as siRNAs and thereby protect them from Dicer and RISC recognition (Chao et al., 2005; Fenner et al., 2006, 2007; Li et al., 2002; Lingel et al., 2005). Therefore, this suppressor should be functional irrespective of the organism. However, in *Tribolium* the capability of B2 to inhibit the RNAi pathway remains still unclear. While FHV B2 did not show suppression of tGFP RNAi which was driven by the Gal4/UAS system (Fig. 4.8), it displayed slight inhibition of *Tc-ebony* RNAi (Fig. 4.9 E, F). It seems that B2 interferes to some extent with the RNAi mechanism but this interference might be not sufficient enough. Likewise, in *Drosophila* the *Flock House virus* is less virulent than the *Cricket Paralysis virus* which might correlate with a less potent RNAi suppressor of FHV (Wang et al., 2006). Considering the mode of action of B2 it is quite likely that high amounts of B2 are needed to bind all applied dsRNAs or siRNAs to block RNAi against highly expressed genes. Hence, either the B2 expression level was insufficient to efficiently disrupt RNAi or the expression levels of target genes were too high to be completely rescued or both. The applied dsRNA concentration might be a further important factor. Indeed, silencing of *Tc-ebony* in B2 expressing animals using 1.6µg/µl concentrated dsRNA resulted in a minor interference with RNAi (Fig. 4.9 E, F). The knockdown of tGFP, however, using 2.5µg/µl concentrated dsRNA was not suppressed by this VSR (Fig. 4.8). To test

whether the concentration was actually responsible for these varying outcomes, the different approaches should be repeated with decreased dsRNA concentrations.

Besides, FHV B2 has also been demonstrated to bind the PAZ domain of Dicer proteins in armyworm cells (Singh et al., 2009). *Tribolium* Dicer-2, which is involved in the RNAi pathway contains a PAZ domain, but this is more diverged compared to the domains of Tc-Dicer-1 and Dm-Dicer-1 (Tomoyasu et al., 2008). Hence, the inefficiency of B2 in *Tribolium* might be a result of the diverged Dicer-2 PAZ domain. However, *Drosophila* Dicer-2 protein is assumed to lack PAZ but FHV B2 is still able to impair the RNAi mechanism in this species (Berry et al., 2009; Lee et al., 2004; van Rij et al., 2006; Tomoyasu et al., 2008; Wang et al., 2006). Therefore, whether the PAZ interaction plays an essential role for B2-mediated RNAi suppression in *Tribolium* and *Drosophila* remains unclear.

The RNAi suppressor of *Drosophila C virus*, DCV1A, functions in a similar way as FHV B2, namely by binding long dsRNAs but not siRNAs (van Rij et al., 2006). In my tests, it did not show any RNAi inhibition activity in *Tribolium* (Fig. 4.7, 4.8, 4.9). This inefficiency might have the same reasons like B2. However, a further reason for the failed RNAi suppression might be the assumed additional ability of the inhibitor to affect the RISC complex assembly (Nayak et al., 2010). The detailed mechanism how DCV1A interferes with RISC in *Drosophila* is still unclear but the interaction proteins/domains/sequences might differ to those of *Tribolium.*

5.2.4 Plant viral RNAi suppressors p38 and p25 might be ineffective in *Tribolium* due to their non-conserved interaction partners

The viral suppressor p38 from the *Turnip Crinkle virus* has been shown to bind Ago-1 of plants by imitating host endogenous GW-containing proteins to recruit Ago proteins (Azevedo et al., 2010). This binding blocks RISC complex assembly and thereby disrupts the RNAi mechanism in plants. While p38 interfered with the RNAi mechanism in animal cell culture, it did not reveal any activity in the fruit fly (Berry et al., 2009; Dunoyer et al., 2004). The same was true for the flour beetle in my experiments (Fig. 4.7, 4.8, 4.9). GW-containing proteins have been demonstrated to be functionally and evolutionarily conserved and are among others necessary for Argonaute binding in several species, including *Drosophila melanogaster* (El-Shami et al., 2007). Both Dm-Ago-1 and Dm-Ago-2 which are involved in the miRNA and

siRNA pathway, respectively, are able to interact with GW proteins (e.g. GW182) (Behm-Ansmant et al., 2006; Schneider et al., 2006). However, despite this interaction of GW domains with Ago proteins and the fact that suppressor p38 reveals GW-repeats and is able to recruit Ago-1 protein of plants there was no visible RNAi suppression activity in insects. Furthermore, the plant as well as fly miRNA pathway seems also not to be affected by p38 (Berry et al., 2009; Dunoyer et al., 2004; Ruiz-Ferrer and Voinnet, 2009; Schott et al., 2012) Hence, either the suppression model of p38 is incorrect or the mechanism of action of p38 is more complex and includes interaction partners which are not conserved between plants and insects.

The RNAi suppression mechanism of p25 from the *Potato virus X* relies on its interference with RNA-dependent RNA polymerase in plants. However, *Drosophila* as well as *Tribolium* seem to lack an RdRP-mediated amplification of RNAi effect (Tomoyasu et al., 2008). This might be the reason for the inefficiency of p25 in suppressing the RNAi mechanism of *Tribolium* (Fig. 4.7, 4.8, 4.9).

5.2.4.1 Further plant VSRs might be more effective

It will be interesting to test further plant viral RNAi suppressors in *Tribolium*, like p15 from the *Peanut Clump virus*, p19 from the *Tomato Bushy Stunt virus* and p21 from the *Beet Yellows virus* which inhibit plant RNAi machinery by siRNA binding and have been demonstrated to efficiently suppress the RNAi mechanism in transgenic *Drosophila* flies without interrupting the miRNA pathway (Berry et al., 2009; Mérai et al., 2006; Vargason et al., 2003; Ye et al., 2003).

5.2.5 Potential reasons for the failure of the Dicer-i line

It has been demonstrated that the knockdown of *Tc-Dicer-2* and a subsequent EGFP dsRNA injection into an enhancer trap line which expresses EGFP in the eyes and the wing primordia, resulted in reduced efficiency of EGFP RNAi. By this method Tc-Dcr-2 has been shown to be involved in the RNAi pathway in *Tribolium* (Tomoyasu et al., 2008). A similar approach was used during this thesis with the difference that *Tc-Dcr-2* was not downregulated by injection but instead, it was constitutively silenced by an inverted-repeat transgene ubiquitously expressed in the

beetles. However, the transgenic Dicer-i line did not produce visible RNAi suppression in *Tribolium* (Fig. 4.9 P, Q). The outcome is unexpected due to the fact that an endogenous gene involved in the RNAi mechanism was targeted in this case. The Dicer-i line carried IR sequences against the 3'UTR of *Tc-Dcr-2* mRNA. This IR construct was driven by a ubiquitously active *Tc-alpha-Tubulin1* promoter (Siebert et al., 2008). However, there is some evidence that *Tc-alpha-Tubulin1* is a rather weak promoter and thereby might be not effective enough to drive strong transgene expression which is necessary to efficiently downregulate the *Tc-Dcr-2* mRNA (Bucher, personal communication). Furthermore, the 3'UTR sequence might be not able to sufficiently downregulate the *Dcr-2* gene. In order to test this, successive dsRNA injections could be performed as described above, in which first dsRNA against 3'UTR of *Tc-Dcr-2* could be injected at different concentrations followed by dsRNA injection against e.g. *Tc-ebony*. In case of negative results (namely no visible suppression of *Tc-ebony* RNAi), another dsRNA fragment of the coding sequence of *Tc-Dcr-2* could be used. In case that high dsRNA concentrations of 3'UTR of *Tc-Dcr-2* are required to suppress *Tc-ebony* RNAi, the expression level of the 3'UTR-IR construct should be increased. Therefore, *Tc-alpha-Tubulin1* could be replaced by *Tc-polyubiquitin* promoter which might be more efficient for transgene expression (Lorenzen et al., 2002b). A further possible reason for the failure of Dicer-i line might be an unsuitable intron spacer which was used to stabilize the inverted repeat sequences during cloning procedure and to enhance nuclear export of the transgene (Kalidas and Smith, 2002; Lee and Carthew, 2003; Llopart et al., 2002; Maniatis and Reed, 2002; Reichhart et al., 2002). Upon transgene expression, the intron should be spliced out thus enabling the folding of IRs into hairpin-loops. Incorrect splicing might account for inefficient hairpin folding, leading to a non-functional transgenic line.

5.2.6 The effectiveness of most RNAi suppressors remains unclear and needs further analyses

Many experiments could be performed in order to investigate the respective reasons for the non-functionality of the most VSRs and the Dicer-i line in *Tribolium*. For instance, by RT-qPCR slight RNAi suppression activity of VSRs or the Dicer-i line could be measured. Upon RNAi, expression changes of e.g. *Tc-ebony* mRNA in VSR or *Tc-Dcr-2*-IR (of Dicer-i line) producing beetles in relation to the controls might thus

be validated. In case that some of the RNAi inhibitors suppress the RNAi mechanism but this suppression is insufficient for clear results, the efficiency of these inhibitors could be enhanced by e.g. increasing the level of inhibitor expression. To this end, the ubiquitously active promoter *Tc-polyubiquitin* could be used (Lorenzen et al., 2002b).

Alternatively, the potency of the inhibitors could be enhanced by viral synergism. It has been demonstrated in plants and insects that co-infection of hosts with two distinct viruses can elicit more severe disease symptoms than those induced by single infections. These synergistic effects seem to depend among others on viral inhibitors which might act as synergism genes (Berry et al., 2009; Pruss et al., 1997; van Rij et al., 2006).

However, given that the RNAi suppressor CrPV1A efficiently inhibits the RNAi pathway in *Tribolium,* the most important experiment which is required for further application of CrPV1A is to analyze whether this VSR can cross cell boundaries. The potential tests are described in section 5.2.1.1

5.3 Establishment of a tool for temporal regulation of RNAi

5.3.1 Start of the RNAi effect after injection is too slow to separate sequential gene functions

RNAi is an effective tool for gene functional studies in *Tribolium*. However, due to the multifunctional roles of some genes, their downregulation can induce pleiotropic effects which can be obstructive for the analysis of gene functions. Temporal regulation of RNAi, e.g. by varying the timing of RNAi onset, might circumvent such unwanted effects. For instance, the transcription factor *cap'n'collar* (*cnc*) is an important gene for labrum development in different organisms including *Tribolium* (Birkan et al., 2011; Kittelmann et al., 2013; Mohler et al., 1995). However, pupal RNAi against *Tc-cnc* led to beetle sterility, indicating that *Tc-cnc* is also involved in gonad development or oogenesis. Silencing of *Tc-cnc* in adult flour beetles allowed to overcome this sterility phenotype (Kittelmann et al., 2013). Nevertheless, staged injection of dsRNA is not able to prevent all undesired gene functions as demonstrated by embryonic RNAi against the segmentation gene *Tc-prd* in this study. Downregulation of *Tc-prd* produces a strong pair-rule phenotype with defective

gnathal, thoracic (anterior) and abdominal (posterior) segments (Fig. 4.11 B, Fig. 4.14 B) (Choe and Brown, 2007; Choe et al., 2006). Hence, for test conditions anterior segmentation defects were defined as to circumvent and posterior defects were aimed to be affected by RNAi.

However, by varying the time of dsRNA injection into staged embryos, it was not possible to induce a sharp RNAi pulse i.e. all segments were affected in a similar way either strongly in early injections or weakly in later injected embryos (Fig. 4.11 B). The same was true when embryonic development was slowed down at lower incubation temperature (Fig. 4.11 B). A likely explanation for this outcome is that the RNAi effect starts too slowly to solely affect the posterior elongation without affecting anterior segmentation. The slowdown of embryonic development might also slow down the kinetics of the RNAi machinery. Hence, temporal control over RNAi by the timing of dsRNA injection is only able to separate gene functions which are separated by extended developmental time.

5.3.2 Heat shock inducible CrPV1A activation: General considerations

The heat shock controlled activation of CrPV1A expression might be a more effective way for temporal RNAi regulation because the production of this VSR is assumed to be a fast process. For instance, it has been shown on cultured *Drosophila* S2 cells transfected with CrPV virus, that the viral RNA reached maximal levels within 6h post infection (Garrey et al., 2010; Wilson et al., 2000). This rapid virus amplification might correlate with a rapid VSR activity which is responsible for the protection of viral mRNA from host RNAi defense mechanism.

Before application, the time point for the onset of VSR expression (VSR pulse) should be optimized depending on the studied process due to the unknown delay between the heat shock activation and the production of functional CrPV1A protein. In order to test how much time it takes from heat shock to regained gene expression (e.g. of *Tc-prd*), RNAi embryos could be heat shocked and subsequently fixed for in-situ hybridization at different time points after heat shock. This treatment will give information about the lag between the heat shock pulse and the existence of a functional CrPV1A protein.

Some side effects should be considered when performing heat shocks for temporal regulation over the onset of CrPV1A expression. For example, in the locust,

Schistocerca gregaria, as well as in *Drosophila* heat shocks of early embryos resulted in disrupted abdominal segments, indicating either an interference with the segmentation process or deletion of already formed segment primordia (Eberlein, 1985; Mee and French, 1986a, 1986b). Moreover, it has been demonstrated that heat shocks of mutant *Drosophila* embryos for the pair-rule gene *hairy* increased the frequency of *hairy* phenotype, suggesting a more severe effect produced by this treatment (Eberlein, 1985).

I observed the same situation in *Tribolium.* When *Tc-prd* was used as a test gene, some heat shocked, transgenic RNAi embryos showed a completely disrupted segmentation phenotype, called "no segmentation" (Fig. 4.13). This phenotype could be a side effect of *Tc-prd* RNAi combined with the heat shock treatment itself because it appears only in this treatment in significant amounts. Heat shock procedure in transgenic, non-injected hs control embryos also led to a disrupted abdomen (Fig. 4.13) but the additional knockdown of the segmentation gene *Tc-prd* might increase this effect in prdRNAi;+hs embryos as it was reported for mutant *Drosophila* embryos. Therefore, positive and negative controls should always be included when the heat shock inducible CrPV1A line (hsCrPVi) is used for gene functional studies.

5.3.3 Temporal RNAi regulation by CrPV1A activation is possible

By activating the CrPV1A expression via heat shock during embryonic GZ elongation, it was possible to terminate the RNAi effect of a previously silenced *Tc-prd* gene during the segmentation process. This RNAi termination resulted in the expected gnathal and thoracic defects but rescued abdominal segmentation due to the regained *Tc-prd* expression (Fig. 4.13 and Fig. 4.14 D). Restored abdomen was observed in large quantities in those cuticles that were heat shocked at the embryonic age of 10-15h (Fig. 4.13, Fig. 4.14 D). However, many cuticles also displayed 4-5 AS like the non-heat shocked *Tc-prd* RNAi control (Fig. 4.13, Fig. 4.14 B-C). This phenotype could be explained on the one hand by the non-homozygous state of hsCrPVi lines, producing a low percentage (at most 25%) of progeny without transgene CrPV1A. On the other hand, VSR activation might have been too late for a rescue effect in older embryonic stages. This explanation is in line with the results of

11-16h and 12-17h old RNAi embryos which showed a decreased number of cuticles with recovered abdomen post heat shock (Fig. 4.13).

As egg collections of embryos aged 10-15h showed germ rudiment to elongating germ band stages with at most nine *Tc-wg* stripes (three established abdominal segments), the VSR pulse should be performed in germ band stages (embryos with up to six *Tc-wg* stripes) before *Tc-wg* expression arise in the abdominal segment anlagen (Fig. 4.12). The early VSR activation in this experiment is important due to the rapid segmentation process in *Tribolium* which establishes all segment anlagen within seven hours post germ rudiment formation (at 32°C) (Fig. 4.12) and the unknown delay between heat shock and CrPV1A production as described in section 5.3.2. Hence, further heat shock tests with *Tc-prd* RNAi embryos at the age of approximately 10-13h as well as homozygous hsCrPVi lines are necessary to achieve higher cuticle amounts with rescued segmentation.

In summary, the heat shock inducible CrPV1A line can be used to temporally regulate the RNAi effect of previously silenced genes, thereby enabling a more detailed investigation of genes involved in different processes.

5.3.4 Prospective application of hsCrPVi line to analyze genes involved in abdominal patterning

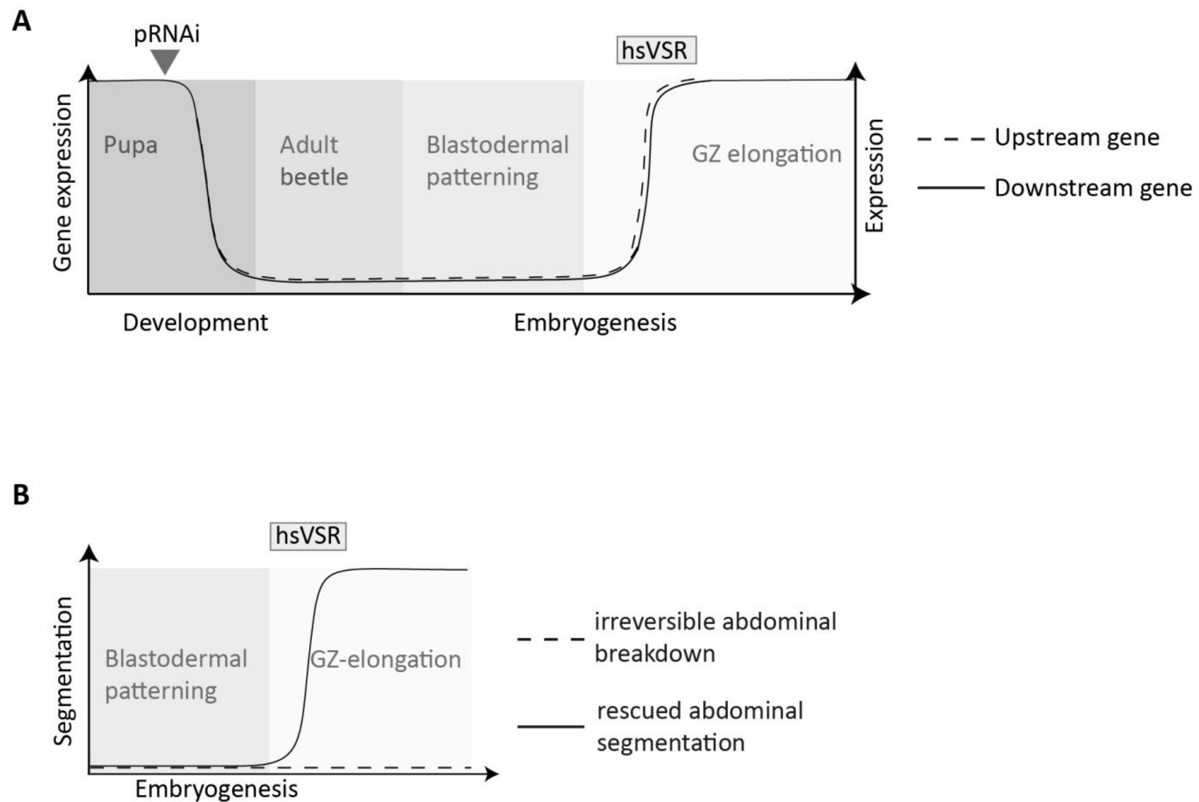
The possibility to regain the expression of silenced genes by temporally defined VSR activation allows a detailed analysis of genes involved e.g. in the GZ segmentation process of *Tribolium*.

Unlike the long germ insect *Drosophila melanogaster*, where all body segments form simultaneously in a syncytial blastoderm, in the short germ insect *Tribolium* only anterior segments are built at the blastodermal stage. The abdominal segments form sequentially from a posterior GZ during germ band elongation. The detailed mechanism is not well understood, but there is some evidence that a segmentation clock determines the segment formation during elongation similar to vertebrates (Choe et al., 2006; Sarrazin et al., 2012). Moreover, a dynamic wave like expression of the primary pair-rule gene, *Tc-even-skipped*, has been demonstrated at the blastoderm stages, indicating that a clock mechanism is employed in the blastoderm patterning as well (El-Sherif et al., 2012). Potential candidates for the vertebrate-like clock and wavefront model are the three *Tribolium* primary pair-rule genes which

might represent the clock, namely *Tc-even-skipped*, *Tc-runt* and *Tc-odd-skipped.* These genes have been described to regulate one another as well as their downstream targets, the secondary pair-rule genes *Tc-paired* and *Tc-sloppy-paired*, in a three-gene circuit (Choe and Brown, 2007; Choe et al., 2006). Candidates for the wavefront are *caudal*, some members of the Wnt signalling pathway (*WntD/8*, *arrow*, *Frizzled-1*, *Frizzled-2*) or members of the FGF signalling pathway (Beermann et al., 2011; Bolognesi et al., 2008, 2009; Copf et al., 2004; El-Sherif et al., 2014). Despite their assumed different roles, they all lead to a segmentation breakdown when silencing.

The knockdown of these candidate genes and a subsequent expression rescue during GZ elongation by hsCrPVi activation might clarify whether there is an upstream factor that can re-induce the GZ or whether the segmentation breakdown is irreversible.

Parental RNAi of assumed upstream genes (e.g. *caudal* or Wnt pathway) would downregulate the respective gene expression in the blastodermal stages of embryonic progeny, resulting in affected anterior segmentation (Fig. 5.1 A). The following VSR activation during GZ elongation should rescue the expression (Fig. 5.1 A) but not the abdominal segmentation due to the upstream function of these genes (Fig. 5.1 B dashed line). On the other hand, RNAi against assumed downstream targets (e.g. *eve*, *odd*, *runt*) should lead to temporary abdomen disruption and should be recovered by RNAi suppressor activation during GZ elongation as it has been demonstrated for the downstream, secondary pair-rule gene *Tc-prd* (Fig. 5.1 B solid line).



5.1 Model for the investigation of genes involved in GZ elongation. (A) Gene expression changes post RNAi and VSR activation. The x-axis represents organism development as well as the offspring embryogenesis. The y-axis represents the expression of genes which are involved in embryonic development. Parental RNAi against candidate genes involved in GZ elongation will result in a downregulation of the respective gene expression (decreasing lines). Heat shock mediated VSR activation (indicated by bars) will rescue the gene expressions of both downstream and upstream genes (increasing lines). (B) Different segmentation phenotypes after VSR activation. The x-axis represents the embryogenesis. The y-axis represents the segmentation after gene silencing. hsVSR activation should rescue abdominal segmentation when the silenced genes are downstream in the GZ regulatory cascade (solid line) but should lead to an irreversible abdominal breakdown when silenced genes are upstream factors (dashed line).

5.4 Concluding remarks

I showed that the viral protein CrPV1A can be successfully used in *Tribolium* to block the RNAi pathway without impairing the development or viability of the organisms. Ubiquitous and constitutive expression of CrPV1A in all developmental stages is necessary to strengthen the hypothesis that the suppressor protein does not influence the miRNA pathway. Furthermore, RT-qPCR analyses should answer the question concerning the ability of other VSRs to suppress the RNAi pathway in *Tribolium*.

The establishment of a heat shock inducible CrPV1A line has been demonstrated to temporally regulate the RNAi pathway. Activation of CrPV1A was able to terminate the RNAi response of a previously silenced segmentation gene and even to rescue the gene specific phenotype. By this temporally controlled tool, investigations of further genes involved in the GZ maintenance are possible which will elucidate their genetic interactions in the segmentation process. Moreover, by using CrPV1A spatially regulated RNAi silencing might be possible which will further strengthen *Tribolium* as an important model organism for gene functional studies.

6 Literature

Akiyama-Oda, Y., and Oda, H. (2006). Axis specification in the spider embryo: dpp is required for radial-to-axial symmetry transformation and sog for ventral patterning. Development *133*, 2347–2357.

Aliyari, R., Wu, Q., Li, H.-W., Wang, X.-H., Li, F., Green, L.D., Han, C.S., Li, W.-X., and Ding, S.-W. (2008). Mechanism of induction and suppression of antiviral immunity directed by small RNAs in Drosophila. Cell Host Microbe *4*, 387–397.

Almond, J.B., and Cohen, G.M. (2002). The proteasome: a novel target for cancer chemotherapy. Leukemia *16*, 433–443.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J Mol Biol *215*, 403–410.

Aravin, A.A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., Gaasterland, T., Meyer, J., and Tuschl, T. (2003). The Small RNA Profile during Drosophila melanogaster Development. Developmental Cell *5*, 337–350.

Azevedo, J., Garcia, D., Pontier, D., Ohnesorge, S., Yu, A., Garcia, S., Braun, L., Bergdoll, M., Hakimi, M.A., Lagrange, T., et al. (2010). Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. Genes Dev. *24*, 904–915.

Bashirullah, A., Cooperstock, R.L., and Lipshitz, H.D. (2001). Spatial and temporal control of RNA stability. Proc Natl Acad Sci U S A *98*, 7025–7028.

Baum, J.A., Bogaert, T., Clinton, W., Heck, G.R., Feldmann, P., Ilagan, O., Johnson, S., Plaetinck, G., Munyikwa, T., Pleau, M., et al. (2007). Control of coleopteran insect pests through RNA interference. Nat. Biotechnol. *25*, 1322–1326.

Baxter, S.W., Zhao, J.-Z., Gahan, L.J., Shelton, A.M., Tabashnik, B.E., and Heckel, D.G. (2005). Novel genetic basis of field-evolved resistance to Bt toxins in Plutella xylostella. Insect Molecular Biology *14*, 327–334.

Bayne, E.H., Rakitina, D.V., Morozov, S.Y., and Baulcombe, D.C. (2005). Cell-to-cell movement of Potato Potexvirus X is dependent on suppression of RNA silencing. The Plant Journal *44*, 471–482.

Beermann, A., Pruhs, R., Lutz, R., and Schroder, R. (2011). A context-dependent combination of Wnt receptors controls axis elongation and leg development in a short germ insect. Development *138*, 2793–2805.

Behm-Ansmant, I., Rehwinkel, J., and Izaurralde, E. (2006). MicroRNAs Silence Gene Expression by Repressing Protein Expression and/or by Promoting mRNA Decay. Cold Spring Harb Symp Quant Biol *71*, 523–530.

Berghammer, A.J., Klingler, M., and Wimmer, E.A. (1999). A universal marker for transgenic insects. Nature *402*, 370–371.

Berghammer, A.J., Weber, M., Trauner, J., and Klingler, M. (2009). Red flour beetle (Tribolium) germline transformation and insertional mutagenesis. Cold Spring Harb Protoc *2009*, pdb.prot5259.

Bernstein, E., Caudy, A.A., Hammond, S.M., and Hannon, G.J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature *409*, 363–366.

Berry, B., Deddouche, S., Kirschner, D., Imler, J.-L., and Antoniewski, C. (2009). Viral Suppressors of RNA Silencing Hinder Exogenous and Endogenous Small RNA Pathways in Drosophila. PLoS ONE *4*, e5866.

Birkan, M., Schaeper, N.D., and Chipman, A.D. (2011). Early patterning and blastodermal fate map of the head in the milkweed bug Oncopeltus fasciatus. Evol. Dev. *13*, 436–447.

Birmingham, A., Anderson, E.M., Reynolds, A., Ilsley-Tyree, D., Leake, D., Fedorov, Y., Baskerville, S., Maksimova, E., Robinson, K., Karpilow, J., et al. (2006). 3′ UTR seed matches, but not overall identity, are associated with RNAi off-targets. Nat Meth *3*, 199–204.

Bolognesi, R., Farzana, L., Fischer, T.D., and Brown, S.J. (2008). Multiple Wnt genes are required for segmentation in the short-germ embryo of Tribolium castaneum. Curr. Biol. *18*, 1624–1629.

Bolognesi, R., Fischer, T.D., and Brown, S.J. (2009). Loss of Tc-arrow and canonical Wnt signaling alters posterior morphology and pair-rule gene expression in the short-germ insect, Tribolium castaneum. Dev Genes Evol *219*, 369–375.

Bortolamiol, D., Pazhouhandeh, M., Marrocco, K., Genschik, P., and Ziegler-Graff, V. (2007). The Polerovirus F Box Protein P0 Targets ARGONAUTE1 to Suppress RNA Silencing. Current Biology *17*, 1615–1621.

Brown, A.E., and Grossman, S.H. (2004). The mechanism and modes of inhibition of arginine kinase from the cockroach (Periplaneta americana). Arch. Insect Biochem. Physiol. *57*, 166–177.

Brown, S.J., Mahaffey, J.P., Lorenzen, M.D., Denell, R.E., and Mahaffey, J.W. (1999). Using RNAi to investigate orthologous homeotic gene function during development of distantly related insects. Evol Dev *1*, 11–15.

Brown, S.J., Shippy, T.D., Miller, S., Bolognesi, R., Beeman, R.W., Lorenzen, M.D., Bucher, G., Wimmer, E.A., and Klingler, M. (2009). The red flour beetle, Tribolium castaneum (Coleoptera): a model for studies of development and pest biology. Cold Spring Harb Protoc *2009*, pdb emo126.

Bucher, G., and Klingler, M. (2004). Divergent segmentation mechanism in the short germ insect Tribolium revealed by giant expression and function. Development *131*, 1729–1740.

Bucher, G., and Wimmer, E.A. (2005). Beetle a-head. B.I.F Futura *20*, 164–169.

Bucher, G., Scholten, J., and Klingler, M. (2002). Parental RNAi in Tribolium (Coleoptera). Curr Biol *12*, R85–R86.

Campbell, G., Weaver, T., and Tomlinson, A. (1993). Axis specification in the developing Drosophila appendage: the role of wingless, decapentaplegic, and the homeobox gene aristaless. Cell *74*, 1113–1123.

Carmell, M.A., and Hannon, G.J. (2004). RNase III enzymes and the initiation of gene silencing. Nat. Struct. Mol. Biol. *11*, 214–218.

Carmell, M.A., Xuan, Z., Zhang, M.Q., and Hannon, G.J. (2002). The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev. *16*, 2733–2742.

Chamberlin, M. (1997). Mitochondrial arginine kinase in the midgut of the tobacco hornworm (Manduca sexta). J Exp Biol *200*, 2789–2796.

Chao, J.A., Lee, J.H., Chapados, B.R., Debler, E.W., Schneemann, A., and Williamson, J.R. (2005). Dual modes of RNA-silencing suppression by Flock House virus protein B2. Nat. Struct. Mol. Biol. *12*, 952–957.

Chapman, E.J., Prokhnevsky, A.I., Gopinath, K., Dolja, V.V., and Carrington, J.C. (2004). Viral RNA silencing suppressors inhibit the microRNA pathway at an intermediate step. Genes Dev. *18*, 1179–1186.

Chen, Q., Ding, Q., Thorpe, J., Dohmen, R.J., and Keller, J.N. (2005). RNA interference toward UMP1 induces proteasome inhibition in Saccharomyces cerevisiae: evidence for protein oxidation and autophagic cell death. Free Radical Biology and Medicine *38*, 226–234.

Chiu, M.-H., Chen, I.-H., Baulcombe, D.C., and Tsai, C.-H. (2010). The silencing suppressor P25 of Potato virus X interacts with Argonaute1 and mediates its degradation through the proteasome pathway. Molecular Plant Pathology *11*, 641–649.

Choe, C.P., and Brown, S.J. (2007). Evolutionary flexibility of pair-rule patterning revealed by functional analysis of secondary pair-rule genes, paired and sloppy-paired in the short-germ insect, Tribolium castaneum. Dev Biol *302*, 281–294.

Choe, C.P., Miller, S.C., and Brown, S.J. (2006). A pair-rule gene circuit defines segments sequentially in the short-germ insect Tribolium castaneum. Proc. Natl. Acad. Sci. U.S.A. *103*, 6560–6564.

Chu, M., Desvoyes, B., Turina, M., Noad, R., and Scholthof, H.B. (2000). Genetic Dissection of Tomato Bushy Stunt Virus p19-Protein-Mediated Host-Dependent Symptom Induction and Systemic Invasion. Virology *266*, 79–87.

Ciechanover, A. (1994). The ubiquitin-proteasome proteolytic pathway. Cell *79*, 13–21.

Cohen, B., Simcox, A.A., and Cohen, S.M. (1993). Allocation of the thoracic imaginal primordia in the Drosophila embryo. Development *117*, 597–608.

Copf, T., Schroder, R., and Averof, M. (2004). Ancestral role of caudal genes in axis elongation and segmentation. Proceedings of the National Academy of Sciences of the United States of America *101*, 17711–17715.

Cormack, B.P., Valdivia, R.H., and Falkow, S. (1996). FACS-optimized mutants of the green fluorescent protein (GFP). Gene *173*, 33–38.

Coumoul, X., Shukla, V., Li, C., Wang, R.-H., and Deng, C.-X. (2005). Conditional knockdown of Fgfr2 in mice using Cre-LoxP induced RNA interference. Nucleic Acids Res. *33*, e102.

Crawford, L.J., Walker, B., and Irvine, A.E. (2011). Proteasome inhibitors in cancer therapy. J Cell Commun Signal *5*, 101–110.

Dietzel, G., Chen, D., Schnorrer, F., Su, K., Barinova, Y., Fellner, M., Gasser, B., Kinsey, K., Oppel, S., Scheiblauer, S., et al. (2007). A genome-wide transgenic RNAi library for conditional gene inactivation in Drosophila. Nature *448*, 151–157.

Ding, S.-W., and Voinnet, O. (2007). Antiviral Immunity Directed by Small RNAs. Cell *130*, 413–426.

Dönitz, J., Schmitt-Engel, C., Grossmann, D., Gerischer, L., Tech, M., Schoppmeier, M., Klingler, M., and Bucher, G. (2015). iBeetle-Base: a database for RNAi phenotypes in the red flour beetle Tribolium castaneum. Nucleic Acids Res. *43*, D720–D725.

Duffy, J.B. (2002). GAL4 system in drosophila: A fly geneticist's swiss army knife. Genesis *34*, 1–15.

Dunoyer, P., Lecellier, C.-H., Parizotto, E.A., Himber, C., and Voinnet, O. (2004). Probing the MicroRNA and Small Interfering RNA Pathways with Virus-Encoded Suppressors of RNA Silencing. Plant Cell *16*, 1235–1250.

Dutton, A., Klein, H., Romeis, J., and Bigler, F. (2002). Uptake of Bt-toxin by herbivores feeding on transgenic maize and consequences for the predator Chrysoperla carnea. Ecological Entomology *27*, 441–447.

Eberlein, S. (1985). Stage specific embryonic defects following heat shock in Drosophila. Dev. Genet. *6*, 179–197.

Elbashir, S.M., Martinez, J., Patkaniowska, A., Lendeckel, W., and Tuschl, T. (2001). Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. EMBO J. *20*, 6877–6888.

El-Shami, M., Pontier, D., Lahmy, S., Braun, L., Picart, C., Vega, D., Hakimi, M.-A., Jacobsen, S.E., Cooke, R., and Lagrange, T. (2007). Reiterated WG/GW motifs form functionally and evolutionarily conserved ARGONAUTE-binding platforms in RNAi-related components. Genes Dev. *21*, 2539–2544.

El-Sherif, E., Averof, M., and Brown, S.J. (2012). A segmentation clock operating in blastoderm and germband stages of Tribolium development. Development *139*, 4341–4346.

El-Sherif, E., Zhu, X., Fu, J., and Brown, S.J. (2014). Caudal regulates the spatiotemporal dynamics of pair-rule waves in Tribolium. PLoS Genet. *10*, e1004677.

Fairbairn, D.J., Cavallaro, A.S., Bernard, M., Mahalinga-Iyer, J., Graham, M.W., and Botella, J.R. (2007). Host-delivered RNAi: an effective strategy to silence genes in plant parasitic nematodes. Planta *226*, 1525–1533.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. Evolution *39*, 783–791.

Fenner, B.J., Goh, W., and Kwang, J. (2006). Sequestration and Protection of Double-Stranded RNA by the Betanodavirus B2 Protein. J. Virol. *80*, 6822–6833.

Fenner, B.J., Goh, W., and Kwang, J. (2007). Dissection of Double-Stranded RNA Binding Protein B2 from Betanodavirus. J. Virol. *81*, 5449–5459.

Ffrench-Constant, R.H. (2013). The molecular genetics of insecticide resistance. Genetics *194*, 807–815.

Ffrench-Constant, R.H. (2014). Insecticide resistance comes of age. Genome Biol. *15*, 106.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature *391*, 806–811.

Fortier, E., and Belote, J.M. (2000). Temperature-dependent gene silencing by an expressed inverted repeat in Drosophila. Genesis *26*, 240–244.

Fuente, J. de la, Almazán, C., Naranjo, V., Blouin, E.F., and Kocan, K.M. (2006). Synergistic effect of silencing the expression of tick protective antigens 4D8 and Rs86 in Rhipicephalus sanguineus by RNA interference. Parasitol Res *99*, 108–113.

Garrey, J.L., Lee, Y.-Y., Au, H.H.T., Bushell, M., and Jan, E. (2010). Host and Viral Translational Mechanisms during Cricket Paralysis Virus Infection. J. Virol. *84*, 1124–1138.

Gassmann, A.J., Petzold-Maxwell, J.L., Keweshan, R.S., and Dunbar, M.W. (2011). Field-Evolved Resistance to Bt Maize by Western Corn Rootworm. PLoS ONE *6*, e22629.

Gelbart, W.M., Crosby, M.A., Matthews, B., Rindone, W.P., Chillemi, J., Twombly, S.R., Emmert, D., Ashburner, M., Drysdale, R.A., Whitfield, E., et al. (1997). FlyBase: a Drosophila database. The FlyBase consortium. Nucleic Acids Research *25*, 63–66.

Gierut, J.J., Jacks, T.E., and Haigis, K.M. (2014). Strategies to Achieve Conditional Gene Mutation in Mice. Cold Spring Harb Protoc *2014*, 339–349.

Giordano, E., Rendina, R., Peluso, I., and Furia, M. (2002). RNAi triggered by symmetrically transcribed transgenes in Drosophila melanogaster. Genetics *160*, 637–648.

Gomariz-Zilber, E., and Thomas-Orillard, M. (1993). Drosophila C virus and Drosophila hosts: a good association in various environments. Journal of Evolutionary Biology *6*, 677–689.

Gomariz-Zilber, E., Poras, M., and Thomas-Orillard, M. (1995). Drosophila C Virus: Experimental Study of infectious Yields and UnderLying Pathology in Drosophila melanogaster Laboratory Populations. Journal of Invertebrate Pathology *65*, 243–247.

Gordon, K.H.J., and Waterhouse, P.M. (2007). RNAi for insect-proof plants. Nat. Biotechnol. *25*, 1231–1232.

Gould, F., Anderson, A., Jones, A., Sumerford, D., Heckel, D.G., Lopez, J., Micinski, S., Leonard, R., and Laster, M. (1997). Initial frequency of alleles for resistance to Bacillus thuringiensis toxins in field populations of Heliothis virescens. PNAS *94*, 3519–3523.

Graveley, B.R., Brooks, A.N., Carlson, J.W., Duff, M.O., Landolin, J.M., Yang, L., Artieri, C.G., van Baren, M.J., Boley, N., Booth, B.W., et al. (2011). The developmental transcriptome of Drosophila melanogaster. Nature *471*, 473–479.

Grossmann, D., Scholten, J., and Prpic, N.-M. (2009). Separable functions of wingless in distal and ventral patterning of the Tribolium leg. Dev Genes Evol *219*, 469–479.

Gu, L., and Knipple, D.C. (2013). Recent advances in RNA interference research in insects: Implications for future insect pest management strategies. Crop Protection *45*, 36–40.

Guo, S., and Kemphues, K.J. (1995). par-1, a gene required for establishing polarity in C. elegans embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell *81*, 611–620.

Habayeb, M.S., Ekengren, S.K., and Hultmark, D. (2006). Nora virus, a persistent virus in Drosophila, defines a new picorna-like virus family. J Gen Virol *87*, 3045–3051.

Hacker, D.L., Petty, I.T.D., Wei, N., and Morris, T.J. (1992). Turnip crinkle virus genes required for RNA replication and virus movement. Virology *186*, 1–8.

Hammond, S.M. (2005). Dicing and slicing: the core machinery of the RNA interference pathway. FEBS Lett. *579*, 5822–5829.

Hammond, S.M., Bernstein, E., Beach, D., and Hannon, G.J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature *404*, 293–296.

Han, D.D., Stein, D., and Stevens, L.M. (2000). Investigating the function of follicular subpopulations during Drosophila oogenesis through hormone-dependent enhancer-targeted cell ablation. Development *127*, 573–583.

Handel, K., Grunfelder, C.G., Roth, S., and Sander, K. (2000). Tribolium embryogenesis: a SEM study of cell shapes and movements from blastoderm to serosal closure. Dev Genes Evol *210*, 167–179.

Heinemeyer, W., Kleinschmidt, J.A., Saidowsky, J., Escher, C., and Wolf, D.H. (1991). Proteinase yscE, the yeast proteasome/multicatalytic-multifunctional proteinase: mutants unravel its function in stress induced proteolysis and uncover its necessity for cell survival. EMBO J *10*, 555–562.

Hershko, A. (1997). Roles of ubiquitin-mediated proteolysis in cell cycle control. Curr. Opin. Cell Biol. *9*, 788–799.

Hilbeck, A., Baumgartner, M., Fried, P.M., and Bigler, F. (1998). Effects of transgenic Bacillus thuringiensis corn-fed prey on mortality and development time of immature Chrysoperla cornea (Neuroptera: Chrysopidae). Environmental Entomology *27*, 480–487.

Hilbeck, A., Moar, W.J., Pusztai-Carey, M., Filippini, A., and Bigler, F. (1999). Prey-mediated effects of Cry1Ab toxin and protoxin and Cry2A protoxin on the predator Chrysoperla carnea. Entomologia Experimentalis et Applicata *91*, 305–316.

Hölzl, H., Kapelari, B., Kellermann, J., Seemüller, E., Sümegi, M., Udvardy, A., Medalia, O., Sperling, J., Müller, S.A., Engel, A., et al. (2000). The Regulatory Complex of Drosophila melanogaster 26s Proteasomes Subunit Composition and Localization of a Deubiquitylating Enzyme. J Cell Biol *150*, 119–130.

Hopkins, T.L., and Kramer, K.J. (1992). Insect Cuticle Sclerotization. Annual Review of Entomology *37*, 273–302.

Horn, C., Schmid, B.G.M., Pogoda, F.S., and Wimmer, E.A. (2002). Fluorescent transformation markers for insect transgenesis. Insect Biochem. Mol. Biol. *32*, 1221–1235.

Horn, C., Offen, N., Nystedt, S., Hacker, U., and Wimmer, E.A. (2003). piggyBac-based insertional mutagenesis and enhancer detection as a tool for functional insect genomics. Genetics *163*, 647–661.

Huang, D.W., Sherman, B.T., and Lempicki, R.A. (2009a). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat Protoc *4*, 44–57.

Huang, D.W., Sherman, B.T., and Lempicki, R.A. (2009b). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. Nucleic Acids Res. *37*, 1–13.

Hughes, C.L., and Kaufman, T.C. (2000). RNAi analysis of Deformed, proboscipedia and Sex combs reduced in the milkweed bug Oncopeltus fasciatus: novel roles for Hox genes in the hemipteran head. Development *127*, 3683–3694.

Huvenne, H., and Smagghe, G. (2010). Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: A review. Journal of Insect Physiology *56*, 227–235.

Jackson, A.L., Burchard, J., Schelter, J., Chau, B.N., Cleary, M., Lim, L., and Linsley, P.S. (2006). Widespread siRNA “off-target” transcript silencing mediated by seed region sequence complementarity. RNA *12*, 1179–1187.

Jansen, R.P. (2001). mRNA localization: message on the move. Nat. Rev. Mol. Cell Biol. *2*, 247–256.

Jin, H., and Zhu, J.-K. (2010). A viral suppressor protein inhibits host RNA silencing by hooking up with Argonautes. Genes Dev. *24*, 853–856.

Kalidas, S., and Smith, D.P. (2002). Novel genomic cDNA hybrids produce effective RNA interference in adult Drosophila. Neuron *33*, 177–184.

Kasim, V., Miyagishi, M., and Taira, K. (2004). Control of siRNA expression using the Cre–loxP recombination system. Nucl. Acids Res. *32*, e66–e66.

Kasschau, K.D., and Carrington, J.C. (2001). Long-Distance Movement and Replication Maintenance Functions Correlate with Silencing Suppression Activity of Potyviral HC-Pro. Virology *285*, 71–81.

Kennerdell, J.R., and Carthew, R.W. (2000). Heritable gene silencing in Drosophila using double-stranded RNA. Nat. Biotechnol. *18*, 896–898.

Ketting, R.F., Fischer, S.E., Bernstein, E., Sijen, T., Hannon, G.J., and Plasterk, R.H. (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev. *15*, 2654–2659.

King, R.W., Deshaies, R.J., Peters, J.M., and Kirschner, M.W. (1996). How proteolysis drives the cell cycle. Science *274*, 1652–1659.

Kittelmann, S., Ulrich, J., Posnien, N., and Bucher, G. (2013). Changes in anterior head patterning underlie the evolution of long germ embryogenesis. Developmental Biology *374*, 174–184.

Kitzmann, P., Schwirz, J., Schmitt-Engel, C., and Bucher, G. (2013). RNAi phenotypes are influenced by the genetic background of the injected strain. BMC Genomics *14*, 5.

Klingler, M. (2004). Tribolium. Current Biology *14*, R639–R640.

Knight, S.W., and Bass, B.L. (2001). A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. Science *293*, 2269–2271.

Kucharski, R., and Maleszka, R. (1998). Arginine kinase is highly expressed in the compound eye of the honey-bee, Apis mellifera. Gene *211*, 343–349.

Kulkarni, M.M., Booker, M., Silver, S.J., Friedman, A., Hong, P., Perrimon, N., and Mathey-Prevot, B. (2006). Evidence of off-target effects associated with long dsRNAs in Drosophila melanogaster cell-based assays. Nat Methods *3*, 833–838.

Kumar, P., Pandit, S.S., and Baldwin, I.T. (2012). Tobacco Rattle Virus Vector: A Rapid and Transient Means of Silencing Manduca sexta Genes by Plant Mediated RNA Interference. PLoS ONE *7*, e31347.

Kwon, D.H., Park, J.H., and Lee, S.H. (2013). Screening of lethal genes for feeding RNAi by leaf disc-mediated systematic delivery of dsRNA in Tetranychus urticae. Pesticide Biochemistry and Physiology *105*, 69–75.

Lakatos, L., Csorba, T., Pantaleo, V., Chapman, E.J., Carrington, J.C., Liu, Y.-P., Dolja, V.V., Calvino, L.F., López-Moya, J.J., and Burgyán, J. (2006). Small RNA binding is a common strategy to suppress RNA silencing by several viral suppressors. The EMBO Journal *25*, 2768–2780.

Lam, G., and Thummel, C.S. (2000). Inducible expression of double-stranded RNA directs specific genetic interference in Drosophila. Curr. Biol. *10*, 957–963.

Lang, A.B., Wyss, C., and Eppenberger, H.M. (1980). Localization of arginine kinase in muscle fibres ofDrosophila melanogaster. J Muscle Res Cell Motil *1*, 147–161.

Lecellier, C.-H., Dunoyer, P., Arar, K., Lehmann-Che, J., Eyquem, S., Himber, C., Saïb, A., and Voinnet, O. (2005). A Cellular MicroRNA Mediates Antiviral Defense in Human Cells. Science *308*, 557–560.

Lee, Y.S., and Carthew, R.W. (2003). Making a better RNAi vector for Drosophila: use of intron spacers. Methods *30*, 322–329.

Lee, Y.S., Nakahara, K., Pham, J.W., Kim, K., He, Z., Sontheimer, E.J., and Carthew, R.W. (2004). Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell *117*, 69–81.

Li, H., Li, W.X., and Ding, S.W. (2002). Induction and Suppression of RNA Silencing by an Animal Virus. Science *296*, 1319–1321.

Li, H., Jiang, W., Zhang, Z., Xing, Y., and Li, F. (2013). Transcriptome Analysis and Screening for Potential Target Genes for RNAi-Mediated Pest Control of the Beet Armyworm, Spodoptera exigua. PLoS ONE *8*, e65931.

Li, J., Chen, Q., Lin, Y., Jiang, T., Wu, G., and Hua, H. (2011a). RNA interference in Nilaparvata lugens (Homoptera: Delphacidae) based on dsRNA ingestion. Pest. Manag. Sci. *67*, 852–859.

Li, X., Zhang, M., and Zhang, H. (2011b). RNA Interference of Four Genes in Adult Bactrocera dorsalis by Feeding Their dsRNAs. PLoS ONE *6*, e17788.

Liao, G.Y., and Xu, B. (2008). Cre recombinase-mediated gene deletion in layer 4 of murine sensory cortical areas. Genesis *46*, 289–293.

Lin, X., Ruan, X., Anderson, M.G., McDowell, J.A., Kroeger, P.E., Fesik, S.W., and Shen, Y. (2005). siRNA-mediated off-target gene silencing triggered by a 7 nt complementation. Nucl. Acids Res. *33*, 4527–4535.

Lingel, A., and Sattler, M. (2005). Novel modes of protein–RNA recognition in the RNAi pathway. Current Opinion in Structural Biology *15*, 107–115.

Lingel, A., Simon, B., Izaurralde, E., and Sattler, M. (2005). The structure of the flock house virus B2 protein, a viral suppressor of RNA interference, shows a novel mode of double-stranded RNA recognition. EMBO Reports *6*, 1149–1155.

Liu, P.Z., and Kaufman, T.C. (2004). hunchback is required for suppression of abdominal identity, and for proper germband growth and segmentation in the intermediate germband insect Oncopeltus fasciatus. Development *131*, 1515–1527.

Liu, F., Wang, X.-D., Zhao, Y.-Y., Li, Y.-J., Liu, Y.-C., and Sun, J. (2015). Silencing the HaAK Gene by Transgenic Plant-Mediated RNAi Impairs Larval Growth of Helicoverpa armigera. Int J Biol Sci *11*, 67–74.

Llopart, A., Comeron, J.M., Brunet, F.G., Lachaise, D., and Long, M. (2002). Intron presence-absence polymorphism in Drosophila driven by positive Darwinian selection. Proc. Natl. Acad. Sci. U.S.A. *99*, 8121–8126.

Lobe, C.G., and Nagy, A. (1998). Conditional genome alteration in mice. Bioessays *20*, 200–208.

Lorenzen, M.D., Brown, S.J., Denell, R.E., and Beeman, R.W. (2002a). Cloning and Characterization of the Tribolium castaneum Eye-Color Genes Encoding Tryptophan Oxygenase and Kynurenine 3-Monooxygenase. Genetics *160*, 225–234.

Lorenzen, M.D., Brown, S.J., Denell, R.E., and Beeman, R.W. (2002b). Transgene expression from the Tribolium castaneum Polyubiquitin promoter. Insect Molecular Biology *11*, 399–407.

Lorenzen, M.D., Kimzey, T., Shippy, T.D., Brown, S.J., Denell, R.E., and Beeman, R.W. (2007). piggyBac-based insertional mutagenesis in Tribolium castaneum using donor/helper hybrids. Insect Mol Biol *16*, 265–275.

Losey, J.E., Rayor, L.S., and Carter, M.E. (1999). Transgenic pollen harms monarch larvae. Nature *399*, 214–214.

Lu, R., Maduro, M., Li, F., Li, H.W., Broitman-Maduro, G., Li, W.X., and Ding, S.W. (2005). Animal virus replication and RNAi-mediated antiviral silencing in Caenorhabditis elegans. Nature *436*, 1040–1043.

Luo, Y., Wang, X., Yu, D., and Kang, L. (2012). The SID-1 double-stranded RNA transporter is not required for systemic RNAi in the migratory locust. RNA Biology *9*, 663–671.

Lynch, J.A., and Desplan, C. (2006). A method for parental RNA interference in the wasp Nasonia vitripennis. Nat Protoc *1*, 486–494.

Lynch, J.A., and Roth, S. (2011). The evolution of dorsal–ventral patterning mechanisms in insects. Genes Dev. *25*, 107–118.

Maniatis, T., and Reed, R. (2002). An extensive network of coupling among gene expression machines. Nature *416*, 499–506.

Manousis, T., and Moore, N.F. (1987). Cricket Paralysis Virus, a Potential Control Agent for the Olive Fruit Fly, Dacus oleae Gmel. Appl. Environ. Microbiol. *53*, 142–148.

Mao, Y.-B., Cai, W.-J., Wang, J.-W., Hong, G.-J., Tao, X.-Y., Wang, L.-J., Huang, Y.-P., and Chen, X.-Y. (2007). Silencing a cotton bollworm P450 monooxygenase gene by plant-mediated RNAi impairs larval tolerance of gossypol. Nat Biotech *25*, 1307–1313.

Martinek, S., and Young, M.W. (2000). Specific genetic interference with behavioral rhythms in Drosophila by expression of inverted repeats. Genetics *156*, 1717–1725.

McGuire, S.E., Le, P.T., Osborn, A.J., Matsumoto, K., and Davis, R.L. (2003). Spatiotemporal rescue of memory dysfunction in Drosophila. Science *302*, 1765–1768.

Mee, J.E., and French, V. (1986a). Disruption of segmentation in a short germ insect embryo II. The structure of segmental abnormalities induced by heat shock. J Embryol Exp Morphol *96*, 267–294.

Mee, J.E., and French, V. (1986b). Disruption of segmentation in a short germ insect embryo I. The location of abnormalities induced by heat shock. J Embryol Exp Morphol *96*, 245–266.

Meister, G., and Tuschl, T. (2004). Mechanisms of gene silencing by double-stranded RNA. Nature *431*, 343–349.

Mérai, Z., Kerényi, Z., Molnár, A., Barta, E., Válóczi, A., Bisztray, G., Havelda, Z., Burgyán, J., and Silhavy, D. (2005). Aureusvirus P14 Is an Efficient RNA Silencing Suppressor That Binds Double-Stranded RNAs without Size Specificity. J. Virol. *79*, 7217–7226.

Mérai, Z., Kerényi, Z., Kertész, S., Magna, M., Lakatos, L., and Silhavy, D. (2006). Double-Stranded RNA Binding May Be a General Plant RNA Viral Strategy To Suppress RNA Silencing. J. Virol. *80*, 5747–5756.

Van Mierlo, J.T., Bronkhorst, A.W., Overheul, G.J., Sadanandan, S.A., Ekström, J.-O., Heestermans, M., Hultmark, D., Antoniewski, C., and van Rij, R.P. (2012). Convergent evolution of argonaute-2 slicer antagonism in two distinct insect RNA viruses. PLoS Pathog. *8*, e1002872.

Van Mierlo, J.T., Overheul, G.J., Obadia, B., van Cleef, K.W.R., Webster, C.L., Saleh, M.-C., Obbard, D.J., and van Rij, R.P. (2014). Novel Drosophila Viruses Encode Host-Specific Suppressors of RNAi. PLoS Pathog *10*, e1004256.

Mignone, F., Gissi, C., Liuni, S., and Pesole, G. (2002). Untranslated regions of mRNAs. Genome Biol *3*, reviews0004.1–reviews0004.10.

Miller, S.C., Brown, S.J., and Tomoyasu, Y. (2008). Larval RNAi in Drosophila? Dev Genes Evol *218*, 505–510.

Miller, S.C., Miyata, K., Brown, S.J., and Tomoyasu, Y. (2012). Dissecting systemic RNA interference in the red flour beetle Tribolium castaneum: parameters affecting the efficiency of RNAi. PLoS ONE *7*, e47431.

Moffat, A.S. (2001). Finding new ways to fight plant diseases. Science *292*, 2270–2273.

Mohler, J., Mahaffey, J.W., Deutsch, E., and Vani, K. (1995). Control of Drosophila head segment identity by the bZIP homeotic gene cnc. Development *121*, 237–247.

Mullins, M.C., Hammerschmidt, M., Haffter, P., and Nüsslein-Volhard, C. (1994). Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. Current Biology *4*, 189–202.

Murray, R.Z., and Norbury, C. (2000). Proteasome inhibitors as anti-cancer agents. Anticancer Drugs *11*, 407–417.

Nagy, A., and Rossant, J. (2001). Chimaeras and mosaics for dissecting complex mutant phenotypes. Int. J. Dev. Biol. *45*, 577–582.

Narva, K.E., Siegfried, B.D., and Storer, N.P. (2013). Transgenic approaches to western corn rootworm control. Adv. Biochem. Eng. Biotechnol. *136*, 135–162.

Nayak, A., Berry, B., Tassetto, M., Kunitomi, M., Acevedo, A., Deng, C., Krutchinsky, A., Gross, J., Antoniewski, C., and Andino, R. (2010). Cricket paralysis virus antagonizes Argonaute 2 to modulate antiviral defense in Drosophila. Nat. Struct. Mol. Biol. *17*, 547–554.

Nicholson, L., Singh, G.K., Osterwalder, T., Roman, G.W., Davis, R.L., and Keshishian, H. (2008). Spatial and Temporal Control of Gene Expression in Drosophila Using the Inducible GeneSwitch GAL4 System. I. Screen for Larval Nervous System Drivers. Genetics *178*, 215–234.

Niu, J., Jian, H., Xu, J., Chen, C., Guo, Q., Liu, Q., and Guo, Y. (2012). RNAi silencing of the Meloidogyne incognita Rpn7 gene reduces nematode parasitic success. Eur J Plant Pathol *134*, 131–144.

Nusslein-Volhard, C. (1994). Of flies and fishes. Science *266*, 572–574.

Obbard, D.J., Jiggins, F.M., Halligan, D.L., and Little, T.J. (2006). Natural Selection Drives Extremely Rapid Evolution in Antiviral RNAi Genes. Current Biology *16*, 580–585.

Oberhofer, G. (2014). A functional analysis of a signaling center of the insect head. Diploma Thesis; Dpt. for Developmental Biology *Georg-August-University Goettingen.*

Okamura, K., Ishizuka, A., Siomi, H., and Siomi, M.C. (2004). Distinct roles for Argonaute proteins in small RNA-directed RNA cleavage pathways. Genes Dev. *18*, 1655–1666.

Osterwalder, T., Yoon, K.S., White, B.H., and Keshishian, H. (2001). A conditional tissue-specific transgene expression system using inducible GAL4. Proc. Natl. Acad. Sci. U.S.A. *98*, 12596–12601.

Parker, J.S., and Barford, D. (2006). Argonaute: A scaffold for the function of short regulatory RNAs. Trends Biochem. Sci. *31*, 622–630.

Pasquinelli, A.E., Hunter, S., and Bracht, J. (2005). MicroRNAs: a developing story. Curr. Opin. Genet. Dev. *15*, 200–205.

Patterson, G., Day, R.N., and Piston, D. (2001). Fluorescent protein spectra. J Cell Sci *114*, 837–838.

Pechmann, M., Khadjeh, S., Turetzek, N., McGregor, A.P., Damen, W.G.M., and Prpic, N.-M. (2011). Novel Function of Distal-less as a Gap Gene during Spider Segmentation. PLoS Genet *7*, e1002342.

Pereira, C.A., Alonso, G.D., Paveto, M.C., Iribarren, A., Cabanas, M.L., Torres, H.N., and Flawiá, M.M. (2000). Trypanosoma cruzi Arginine Kinase Characterization and Cloning A NOVEL ENERGETIC PATHWAY IN PROTOZOAN PARASITES. J. Biol. Chem. *275*, 1495–1501.

Peters, J.-M., Cejka, Z., Harris, J.R., Kleinschmidt, J.A., and Baumeister, W. (1993). Structural Features of the 26 S Proteasome Complex. Journal of Molecular Biology *234*, 932–937.

Piccin, A., Salameh, A., Benna, C., Sandrelli, F., Mazzotta, G., Zordan, M., Rosato, E., Kyriacou, C.P., and Costa, R. (2001). Efficient and heritable functional knock-out of an adult phenotype in Drosophila using a GAL4-driven hairpin RNA incorporating a heterologous spacer. Nucleic Acids Res. *29*, E55–55.

Pitino, M., Coleman, A.D., Maffei, M.E., Ridout, C.J., and Hogenhout, S.A. (2011). Silencing of Aphid Genes by dsRNA Feeding from Plants. PLoS ONE *6*, e25709.

Plus, N., Croizier, G., Reinganum, C., and Scotti, P.D. (1978). Cricket paralysis virus and Drosophila C virus: Serological analysis and comparison of capsid polypeptides and host range. Journal of Invertebrate Pathology *31*, 296–302.

Posnien, N., Schinko, J., Grossmann, D., Shippy, T.D., Konopova, B., and Bucher, G. (2009). RNAi in the red flour beetle (Tribolium). Cold Spring Harb Protoc *2009*, pdb.prot5256.

Posnien, N., Schinko, J.B., Kittelmann, S., and Bucher, G. (2010). Genetics, development and composition of the insect head - A beetle's view. Arthropod Struct Dev *39*, 399–410.

Price, D.R.G., and Gatehouse, J.A. (2008). RNAi-mediated crop protection against insects. Trends Biotechnol. *26*, 393–400.

Pruss, G., Ge, X., Shi, X.M., Carrington, J.C., and Vance, V.B. (1997). Plant viral synergism: the potyviral genome encodes a broad-range pathogenicity enhancer that transactivates replication of heterologous viruses. Plant Cell *9*, 859–868.

Qiu, S., Adema, C.M., and Lane, T. (2005). A computational study of off-target effects of RNA interference. Nucl. Acids Res. *33*, 1834–1847.

Rajagopal, R., Sivakumar, S., Agrawal, N., Malhotra, P., and Bhatnagar, R.K. (2002). Silencing of Midgut Aminopeptidase N of Spodoptera litura by Double-stranded RNA Establishes Its Role asBacillus thuringiensis Toxin Receptor. J. Biol. Chem. *277*, 46849–46851.

Ramaseshadri, P., Segers, G., Flannagan, R., Wiggins, E., Clinton, W., Ilagan, O., McNulty, B., Clark, T., and Bolognesi, R. (2013). Physiological and Cellular Responses Caused by RNAi- Mediated Suppression of Snf7 Orthologue in Western Corn Rootworm (Diabrotica virgifera virgifera) Larvae. PLoS ONE *8*, e54270.

Reichhart, J.-M., Ligoxygakis, P., Naitza, S., Woerfel, G., Imler, J.-L., and Gubb, D. (2002). Splice-activated UAS hairpin vector gives complete RNAi knockout of single or double target transcripts in Drosophila melanogaster. Genesis *34*, 160–164.

Reinganum, C., O'Loughlin, G.T., and Hogan, T.W. (1970). A nonoccluded virus of the field crickets Teleogryllus oceanicus and T. commodus (Orthoptera: Gryllidae). Journal of Invertebrate Pathology *16*, 214–220.

Van Rensburg, J.B.J. (2007). First report of field resistance by the stem borer, Busseola fusca (Fuller) to Bt-transgenic maize. South African Journal of Plant and Soil *24*, 147–151.

Ria, R., Reale, A., and Vacca, A. (2014). Novel agents and new therapeutic approaches for treatment of multiple myeloma. World J Methodol *4*, 73–90.

Richards, S., Gibbs, R.A., Weinstock, G.M., Brown, S.J., Denell, R., Beeman, R.W., Gibbs, R., Beeman, R.W., Brown, S.J., Bucher, G., et al. (2008). The genome of the model beetle and pest Tribolium castaneum. Nature *452*, 949–955.

Van Rij, R.P., Saleh, M.-C., Berry, B., Foo, C., Houk, A., Antoniewski, C., and Andino, R. (2006). The RNA silencing endonuclease Argonaute 2 mediates specific antiviral immunity in Drosophila melanogaster. Genes Dev *20*, 2985–2995.

Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A.L. (1994). Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell *78*, 761–771.

Roignant, J.-Y., Carré, C., Mugat, B., Szymczak, D., Lepesant, J.-A., and Antoniewski, C. (2003). Absence of transitive and systemic pathways allows cell-specific and isoform-specific RNAi in Drosophila. RNA *9*, 299–308.

Roman, G., Endo, K., Zong, L., and Davis, R.L. (2001). P[Switch], a system for spatial and temporal control of gene expression in Drosophila melanogaster. Proc. Natl. Acad. Sci. U.S.A. *98*, 12602–12607.

Ruiz-Ferrer, V., and Voinnet, O. (2009). Roles of Plant Small RNAs in Biotic Stress Responses. Annual Review of Plant Biology *60*, 485–510.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Molecular Biology and Evolution *4*, 406–425.

Saleh, M.-C., Tassetto, M., van Rij, R.P., Goic, B., Gausson, V., Berry, B., Jacquier, C., Antoniewski, C., and Andino, R. (2009). Antiviral immunity in Drosophila requires systemic RNA interference spread. Nature *458*, 346–350.

Sarrazin, A.F., Peel, A.D., and Averof, M. (2012). A segmentation clock with two-segment periodicity in insects. Science *336*, 338–341.

Schinko, J., Hillebrand, K., and Bucher, G. (2012). Heat shock-mediated misexpression of genes in the beetle Tribolium castaneum. Dev Genes Evol. *Dev Genes Evol.*, 287–298.

Schinko, J.B., Weber, M., Viktorinova, I., Kiupakis, A., Averof, M., Klingler, M., Wimmer, E.A., and Bucher, G. (2010). Functionality of the GAL4/UAS system in Tribolium requires the use of endogenous core promoters. BMC Dev Biol *10*, 53.

Schmid, A., Schindelholz, B., and Zinn, K. (2002). Combinatorial RNAi: a method for evaluating the functions of gene families in Drosophila. Trends in Neurosciences *25*, 71–74.

Schmitt-Engel, C., Schultheis, D., Schwirz, J., Ströhlein, N., Troelenberg, N., Majumdar, U., Dao, V.A., Grossmann, D., Richter, T., Tech, M., et al. (2015). The iBeetle large scale RNAi screen reveals novel gene functions for insect development and physiology. Nature Communications.

Schneider, M.D., Najand, N., Chaker, S., Pare, J.M., Haskins, J., Hughes, S.C., Hobman, T.C., Locke, J., and Simmonds, A.J. (2006). Gawky is a component of cytoplasmic mRNA processing bodies required for early Drosophila development. J Cell Biol *174*, 349–358.

Scholthof, H.B. (2006). The Tombusvirus-encoded P19: from irrelevance to elegance. Nat Rev Micro *4*, 405–411.

Schott, G., Mari-Ordonez, A., Himber, C., Alioua, A., Voinnet, O., and Dunoyer, P. (2012). Differential effects of viral silencing suppressors on siRNA and miRNA loading support the existence of two distinct cellular pools of ARGONAUTE1. The EMBO Journal *31*, 2553–2565.

Schröder, R., Beermann, A., Wittkopp, N., and Lutz, R. (2008). From development to biodiversity--Tribolium castaneum, an insect model organism for short germband development. Dev. Genes Evol. *218*, 119–126.

Schwach, F., Vaistij, F.E., Jones, L., and Baulcombe, D.C. (2005). An RNA-Dependent RNA Polymerase Prevents Meristem Invasion by Potato Virus X and Is

Required for the Activity But Not the Production of a Systemic Silencing Signal. Plant Physiol. *138*, 1842–1852.

Scott, J.G., Michel, K., Bartholomay, L.C., Siegfried, B.D., Hunter, W.B., Smagghe, G., Zhu, K.Y., and Douglas, A.E. (2013). Towards the elements of successful insect RNAi. J. Insect Physiol. *59*, 1212–1221.

Siebert, K.S., Lorenzen, M.D., Brown, S.J., Park, Y., and Beeman, R.W. (2008). Tubulin superfamily genes in Tribolium castaneum and the use of a Tubulin promoter to drive transgene expression. Insect Biochem. Mol. Biol. *38*, 749–755.

Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R.H., and Fire, A. (2001). On the role of RNA amplification in dsRNA-triggered gene silencing. Cell *107*, 465–476.

Singh, G., Popli, S., Hari, Y., Malhotra, P., Mukherjee, S., and Bhatnagar, R.K. (2009). Suppression of RNA silencing by Flock house virus B2 protein is mediated through its interaction with the PAZ domain of Dicer. FASEB J *23*, 1845–1857.

Soldatenkov, V.A., and Dritschilo, A. (1997). Apoptosis of Ewing's Sarcoma Cells Is Accompanied by Accumulation of Ubiquitinated Proteins. Cancer Res *57*, 3881–3885.

Spataro, V., Norbury, C., and Harris, A.L. (1998). The ubiquitin-proteasome pathway in cancer. Br. J. Cancer *77*, 448–455.

Struhl, G., and Basler, K. (1993). Organizing activity of wingless protein in Drosophila. Cell *72*, 527–540.

Sullivan, C.S., and Ganem, D. (2005). A Virus-Encoded Inhibitor That Blocks RNA Interference in Mammalian Cells. J. Virol. *79*, 7371–7379.

Tabara, H., Yigit, E., Siomi, H., and Mello, C.C. (2002). The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-box helicase to direct RNAi in C. elegans. Cell *109*, 861–871.

Tabashnik, B.E., Gassmann, A.J., Crowder, D.W., and Carriére, Y. (2008). Insect resistance to Bt crops: evidence versus theory. Nat Biotech *26*, 199–202.

Tabashnik, B.E., Brévault, T., and Carrière, Y. (2013). Insect resistance to Bt crops: lessons from the first billion acres. Nat. Biotechnol. *31*, 510–521.

Takahashi, M., Iwasaki, H., Inoue, H., and Takahashi, K. (2002). Reverse Genetic Analysis of the Caenorhabditis elegans 26S Proteasome Subunits by RNA Interference. Biological Chemistry *383*, 1263–1266.

Tautz, D., Friedrich, M., and Schröder, R. (1994). Insect embryogenesis - what is ancestral and what is derived? Development *Supplement*, 193–199.

Terenius, O., Papanicolaou, A., Garbutt, J.S., Eleftherianos, I., Huvenne, H., Kanginakudru, S., Albrechtsen, M., An, C., Aymeric, J.-L., Barthel, A., et al. (2011). RNA interference in Lepidoptera: An overview of successful and unsuccessful

studies and implications for experimental design. Journal of Insect Physiology *57*, 231–245.

Thakur, N., Upadhyay, S.K., Verma, P.C., Chandrashekar, K., Tuli, R., and Singh, P.K. (2014). Enhanced whitefly resistance in transgenic tobacco plants expressing double stranded RNA of v-ATPase A gene. PLoS ONE *9*, e87235.

Thomas, J.M., Klimstra, W.B., Ryman, K.D., and Heidner, H.W. (2003). Sindbis Virus Vectors Designed To Express a Foreign Protein as a Cleavable Component of the Viral Structural Polyprotein. J. Virol. *77*, 5598–5606.

Tian, H., Peng, H., Yao, Q., Chen, H., Xie, Q., Tang, B., and Zhang, W. (2009). Developmental Control of a Lepidopteran Pest Spodoptera exigua by Ingestion of Bacteria Expressing dsRNA of a Non-Midgut Gene. PLoS ONE *4*, e6225.

Tischler, J., Lehner, B., Chen, N., and Fraser, A.G. (2006). Combinatorial RNA interference in Caenorhabditis elegans reveals that redundancy between gene duplicates can be maintained for more than 80 million years of evolution. Genome Biol. *7*, R69.

Tomoyasu, Y., and Denell, R.E. (2004). Larval RNAi in Tribolium (Coleoptera) for analyzing adult development. Dev Genes Evol *214*, 575–578.

Tomoyasu, Y., Miller, S.C., Tomita, S., Schoppmeier, M., Grossmann, D., and Bucher, G. (2008). Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in Tribolium. Genome Biol *9*, R10.

Trauner, J., Schinko, J., Lorenzen, M.D., Shippy, T.D., Wimmer, E.A., Beeman, R.W., Klingler, M., Bucher, G., and Brown, S.J. (2009). Large-scale insertional mutagenesis of a coleopteran stored grain pest, the red flour beetle Tribolium castaneum, identifies embryonic lethal mutations and enhancer traps. BMC Biol *7*, 73.

Turner, C.T., Davy, M.W., MacDiarmid, R.M., Plummer, K.M., Birch, N.P., and Newcomb, R.D. (2006). RNA interference in the light brown apple moth, Epiphyas postvittana (Walker) induced by double-stranded RNA feeding. Insect Mol. Biol. *15*, 383–391.

Upadhyay, S.K., Chandrashekar, K., Thakur, N., Verma, P.C., Borgio, J.F., Singh, P.K., and Tuli, R. (2011). RNA interference for the control of whiteflies (Bemisia tabaci) by oral route. J Biosci *36*, 153–161.

Vargason, J.M., Szittya, G., Burgyán, J., and Hall, T.M.T. (2003). Size Selective Recognition of siRNA by an RNA Silencing Suppressor. Cell *115*, 799–811.

Van der Velden, A.W., and Thomas, A.A. (1999). The role of the 5’ untranslated region of an mRNA in translation regulation during development. Int. J. Biochem. Cell Biol. *31*, 87–106.

Ventura, A., Meissner, A., Dillon, C.P., McManus, M., Sharp, P.A., Van Parijs, L., Jaenisch, R., and Jacks, T. (2004). Cre-lox-regulated conditional RNA interference from transgenes. Proc Natl Acad Sci U S A *101*, 10380–10385.

Voinnet, O. (2005). Non-cell autonomous RNA silencing. FEBS Letters *579*, 5858–5871.

Voinnet, O., Pinto, Y.M., and Baulcombe, D.C. (1999). Suppression of gene silencing: A general strategy used by diverse DNA and RNA viruses of plants. PNAS *96*, 14147–14152.

Voinnet, O., Lederer, C., and Baulcombe, D.C. (2000). A Viral Movement Protein Prevents Spread of the Gene Silencing Signal in Nicotiana benthamiana. Cell *103*, 157–167.

Wang, X., Luo, H., Chen, H., Duguid, W., and Wu, J. (1998). Role of proteasomes in T cell activation and proliferation. J. Immunol. *160*, 788–801.

Wang, X.-H., Aliyari, R., Li, W.-X., Li, H.-W., Kim, K., Carthew, R., Atkinson, P., and Ding, S.-W. (2006). RNA Interference Directs Innate Immunity Against Viruses in Adult Drosophila. Science *312*, 452–454.

Whangbo, J.S., and Hunter, C.P. (2008). Environmental RNA interference. Trends Genet. *24*, 297–305.

Wieschaus, E., Nüsslein-Volhard, C., and Jürgens, G. (1984). Mutations affecting the pattern of the larval cuticle inDrosophila melanogaster. Wilhelm Roux' Archiv *193*, 296–307.

Wilson, J.E., Powell, M.J., Hoover, S.E., and Sarnow, P. (2000). Naturally Occurring Dicistronic Cricket Paralysis Virus RNA Is Regulated by Two Internal Ribosome Entry Sites. Mol. Cell. Biol. *20*, 4990–4999.

Winston, W.M., Molodowitch, C., and Hunter, C.P. (2002). Systemic RNAi in C. elegans Requires the Putative Transmembrane Protein SID-1. Science *295*, 2456–2459.

Wójcik, C., and DeMartino, G.N. (2002). Analysis of Drosophila 26 S Proteasome Using RNA Interference. J. Biol. Chem. *277*, 6188–6197.

Wójcik, C., Schroeter, D., Wilk, S., Lamprecht, J., and Paweletz, N. (1996). Ubiquitin-mediated proteolysis centers in HeLa cells: indication from studies of an inhibitor of the chymotrypsin-like activity of the proteasome. Eur. J. Cell Biol. *71*, 311–318.

Wright, T.R. (1987). The genetics of biogenic amine metabolism, sclerotization, and melanization in Drosophila melanogaster. Adv. Genet. *24*, 127–222.

Wu, Q.-Y., Li, F., Zhu, W.-J., and Wang, X.-Y. (2007). Cloning, expression, purification, and characterization of arginine kinase from Locusta migratoria manilensis. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology *148*, 355–362.

Wu, W.K.K., Cho, C.H., Lee, C.W., Wu, K., Fan, D., Yu, J., and Sung, J.J.Y. (2010). Proteasome inhibition: a new therapeutic strategy to cancer treatment. Cancer Lett. *293*, 15–22.

Xia, X., Zhou, H., Huang, Y., and Xu, Z. (2006). Allele-specific RNAi selectively silences mutant SOD1 and achieves significant therapeutic benefit in vivo. Neurobiol. Dis. *23*, 578–586.

Xie, Q., and Guo, H.-S. (2006). Systemic antiviral silencing in plants. Virus Research *118*, 1–6.

Xing, S., and Zachgo, S. (2007). Pollen Lethality: A Phenomenon in Arabidopsis RNA Interference Plants. Plant Physiol. *145*, 330–333.

Yang, T.T., Cheng, L., and Kain, S.R. (1996). Optimized codon usage and chromophore mutations provide enhanced sensitivity with the green fluorescent protein. Nucleic Acids Res. *24*, 4592–4593.

Ye, K., Malinina, L., and Patel, D.J. (2003). Recognition of small interfering RNA by a viral suppressor of RNA silencing. Nature *426*, 874–878.

Yigit, E., Batista, P.J., Bei, Y., Pang, K.M., Chen, C.-C.G., Tolia, N.H., Joshua-Tor, L., Mitani, S., Simard, M.J., and Mello, C.C. (2006). Analysis of the C. elegans Argonaute family reveals that distinct Argonautes act sequentially during RNAi. Cell *127*, 747–757.

Yoshimura, T., Kameyama, K., Takagi, T., Ikai, A., Tokunaga, F., Koide, T., Tanahashi, N., Tamura, T., Cejka, Z., Baumeister, W., et al. (1993). Molecular Characterization of the “26S” Proteasome Complex from Rat Liver. Journal of Structural Biology *111*, 200–211.

Yu, B., Chapman, E.J., Yang, Z., Carrington, J.C., and Chen, X. (2006). Transgenically expressed viral RNA silencing suppressors interfere with microRNA methylation in Arabidopsis. FEBS Letters *580*, 3117–3120.

Yusa, K., Zhou, L., Li, M.A., Bradley, A., and Craig, N.L. (2011). A hyperactive piggyBac transposase for mammalian applications. Proc. Natl. Acad. Sci. U.S.A. *108*, 1531–1536.

Zamore, P.D., Tuschl, T., Sharp, P.A., and Bartel, D.P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell *101*, 25–33.

Van der Zee, M., Berns, N., and Roth, S. (2005). Distinct functions of the Tribolium zerknullt genes in serosa specification and dorsal closure. Curr Biol *15*, 624–636.

Zha, W., Peng, X., Chen, R., Du, B., Zhu, L., and He, G. (2011). Knockdown of Midgut Genes by dsRNA-Transgenic Plant-Mediated RNA Interference in the Hemipteran Insect Nilaparvata lugens. PLoS ONE *6*, e20504.

Zhang, B., Kirov, S., and Snoddy, J. (2005). WebGestalt: an integrated system for exploring gene sets in various biological contexts. Nucleic Acids Res. *33*, W741–W748.

Zhang, H., Li, H.-C., and Miao, X.-X. (2013). Feasibility, limitation and possible solutions of RNAi-based technology for insect pest control. Insect Science *20*, 15–30.

Zhang, J., Khan, S.A., Hasse, C., Ruf, S., Heckel, D.G., and Bock, R. (2015). Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids. Science *347*, 991–994.

Zhang, X., Yuan, Y.-R., Pei, Y., Lin, S.-S., Tuschl, T., Patel, D.J., and Chua, N.-H. (2006). Cucumber mosaic virus-encoded 2b suppressor inhibits Arabidopsis Argonaute1 cleavage activity to counter plant defense. Genes Dev. *20*, 3255–3268.

Zhao, Y., Yang, G., Wang-Pruski, G., and You, M. (2008). Phyllotreta striolata (coleoptera: chrysomelidae): arginine kinase cloning and rnai-based pest control. European Journal of Entomology *105*, 815–822.

7 Appendix

7.1 Abbreviations

BB	Bauchbinde
BLAST	Basic Local Alignment Search Tool
bp	base pair
cDNA	complementary DNA
CPB	Colorado potato beetle
Cry	crystal protein
dsRNA	double stranded RNA
ee	empty egg
EGFP	enhanced green fluorescent protein
eRNAi	embryonic RNAi
GFP	green fluorescent protein
GM	genetically modified
GO	Gene Ontology
GW	glycine/tryptophane
GZ	growth zone
hr	hatch rate
hs	heat shock
IR	inverted repeat
lRNAi	larval RNAi
miRNA	microRNA
miRNP	miRNA ribonucleoprotein particle complex
mRNA	messenger RNA
NCBI	National Center for Biotechnology Information
nt	nucleotide
ORF	open reading frame
PAZ domain	Piwi/Argonaute/Zwille domain
PCR	polymerase chain reaction
pRNAi	parental RNAi
RdRP	RNA-dependent RNA polymerase
RISC	RNA-induced silencing complex
RNAi	RNA interference
RT-qPCR	Quantitative real time PCR
SB	*San Bernardino*; a *Tribolium* wild type strain
shRNA	short-hairpin RNA
siRNA	small interfering RNA
TE	transposable element
tGFP	turboGFP
UTR	untranslated region
viRNA	virus-derived RNA

VSR	viral suppressor of RNAi
v^{w}	*vermillion*^{white}; a white-eyed *Tribolium* strain
WCR	western corn rootworm
wt	wild type
An	antenna
AS	abdominal segment
br.	bristles
Lb	labium
Lr	labrum
Md	mandible
Mx	maxilla
no s.	no abdominal segments
T1-T3	thoracic segment 1 to 3
A1-A8	abdominal segment 1 to 8
tracheal.	tracheal openings
Urog.	urogomphi

Organisms:

Aa	*Aedes aegypti*
Am	*Apis mellifera*
Ap	*Acyrthosiphon pisum*
Bt	*Bacillus thuringiensis*
Ce	*Caenorhabditis elegans*
CrPV	*Cricket Paralysis virus*
DCV	*Drosophila C virus*
Dm	*Drosophila melanogaster*
FHV	*Flock House virus*
Mm	*Mus musculus*
PVX	*Potato virus X*
Tc	*Tribolium castaneum*
TCV	*Turnip Crinkle virus*

Genes and proteins:

ago	*Argonaute*
ak	*arginine kinase*
alg-1	*argonaute-like gene 1*
alg-2	*argonaute-like gene 2*
Dcr	*Dicer*
ergo-1	*endogenous RNAi-deficient Argonaute 1*
eve	*even-skipped*
odd	*odd-skipped*
prd	*paired*
Rde-1	*RNAi-defective 1*

rpn7	*regulatory particle non-ATPase 7*
run	*runt*
sid-1	*systemic interference defective-1*
slp	*sloppy-paired*
snf7	*Sucrose non-fermenting 7*
wg	*wingless*
Tc	prefix, if the gene is a *Tribolium* ortholog

FHV B2	Flock House virus B2 protein
CrPV1A	Cricket Paralysis virus 1A protein
DCV1A	Drosophila C virus 1A protein
PVX p25	Potato virus X protein 25
TCV p38	Turnip Crinkle virus protein 38
VP1	Nora virus viral protein 1

7.2 Primers used in this work

Tab. 7.1 Primers for cloning of gene fragments. Primer names, sequences and gene names are shown.

Primer	Sequence	Gene
UFwEbony	CTGTGCTACCAACCCGGAGAGAT	*ebony*
JURvEbony	GTCCATAAGCTCCTTATAGTCCGACT	*ebony*
JUFwL10	CTAAGAGTAAAGTGCAGGAACCAC	*cactus*
JURvL10	TCTGCATGTTCGTAAATCACCAT	*cactus*
JUFwL11	TTGATGACCATCATGGACAG	*Srp54k*
JURvL11	GCAACTGTCGCATCATGTTCT	*Srp54k*
JUFwL44	GTCCAGGACAAACGCATGCAATC	*Ras opposite*
JURvL44	TGTGACTCATACGGCAAGAAGGCA	*Ras opposite*
JUFwL47	GCAAAAAGCTCAGCAGCTAAT	*alpha-snap*
JURvL47	GCAGTTCACCGCCTCGTTAATAT	*alpha-snap*
JUFwL50	GGAAACGGACCGAGTCAC	*shibire*
JURvL50	GAAGACCTTGAGGATCGAC	*shibire*
JUFwL55	CATCTGTCTTTTGCTCGCCTACA	*pp1alpha-96a*
JURvL55	TCAGGATCTGACCACAACAG	*pp1alpha-96a*
JUFwL67	AGACGTGTATGATTGCGACGAT	*Inr-a*
JURvL67	TCTCTCAACTCACGTTTCAAATC	*Inr-a*
JUFwL76	CACTTGCCACATTGACTACAGA	*Hsc70-3*
JURvL76	TCCAGTCACGTCCAATCAGAC	*Hsc70-3*
JUFwL80	GTACGAGCTCATTATCAAGGAC	*Rpn7*
JURvL80	TAAACCGATTCTGATTAGATG	*Rpn7*
FwL82	TAGATCCGAGAGAACAAATGAGA	*Gw*
RvL82	ATGACACCACACTGAACCGTCT	*Gw*
FwL84	GACGAGCAACGCAACTTGAAGA	*Rpt3*
RvL84	GACGTCAGGCTTCTCATCAG	*Rpt3*

Tab. 7.2: Primers for dsRNA synthesis. Primer names, sequences and gene name are shown.

Primer	Sequence	Gene
JUFw_EGFP	taatacgactcactatagggATGGTGAGCAAGGGCGAGGAG	*EGFP*
JURv_EGFP	taatacgactcactatagggTTACTTGTACAGCTCGTCCATG	*EGFP*
JUFw_tGFP	taatacgactcactatagggATGGAGAGCGACGAGAGCG	*tGFP*
JURv_tGFP	taatacgactcactatagggTATTCTTCACCGGCATCTGCATC	*tGFP*

Tab. 7.3: Primers for design of constructs. Primer names, sequences and purpose are shown.

Primer	Sequence	Purpose
JUFw_p25i	ACAGGTACCATGGATATTCTCATCATTAGTTTGA	Cloning of p25 with Acc65I overhang
JURv_p25i	ACAGCGGCCGCTTCTATGTCCCTGCGCGGACAT	Cloning of p25 with NotI overhang
JUFw_p38i	ACAGGTACCATGGAAAATGATCCTAGAGTCCGGAAGT	Cloning of p38 with Acc65I overhang
JURv_p38i	ACAGCGGCCGCTTCTAAATTCTGAGTGCTTGCAAT	Cloning of p38 with NotI overhang
JUFw_DCVi	ACAGGTACCATGGAATCTGATAAAAGTATGGCCTGT	Cloning of DCV1A with Acc65I overhang
JURv_DCVi	ACAGCGGCCGCTTTCCTGATAACAAGAGCAACATCTGA	Cloning of DCV1A with NotI overhang
JUFw_VP1i	ACAGGTACCCCACCAGCAGAGGAGAAAAGGA	Cloning of VP1 with Acc65I overhang
JURv_VP1i	ACAGCGGCCGCTTTTAACATTGTTGTTTCTGCGAGAT	Cloning of VP1 with NotI overhang
JUFw_CrPVi	ACAGGTACCATGTCTTTTCAACAAACAAACAACAAC	Cloning of CrPV1A with Acc65I overhang
JURv_CrPVi	ACAGCGGCCGCTTGAAGGCTCTGCATTCATCATTACT	Cloning of CrPV1A with NotI overhang
JUFw_B2i	ACAGGTACCATGCCAAGCAAACTCGCGCTAATCCAG	Cloning of B2 with Acc65I overhang
JURv_B2i	ACAGCGGCCGCTTCAGTTTTGCGGGTGGGGGGTCA	Cloning of B2 with NotI overhang
UTRFw_Inf	CAACTACCAAGCTAGTAACTTGAAACAGTTTTAATTTGA	Cloning of IRs by InFusion system
UTRRv_Inf	ATCTTCTCTCTGGTATTAACGCACGAAGTCACAATG	Cloning of IRs by InFusion system
UTR2Fw_Inf	ATTTCGATCCAAGTATTAACGCACGAAGTCACAATG	Cloning of IRs by InFusion system
UTR2Rv_Inf	TCCAAACTCATCAGGTAACTTGAAACAGTTTTAATTTGA	Cloning of IRs by InFusion system
IntronFw_Inf	GACTTCGTGCGTTAATACCAGAGAGAAGATCGTGTTAC	Cloning of an intron by InFusion system
IntronRv_Inf	GACTTCGTGCGTTAATACTTGGATCGAAATGCTC	Cloning of an intron by InFusion system
JUFw_pBacL	TATCTCGAGCTTAAGGGGCCCTATCTTAGATCTGAC AATGTTCA	PCR amplification of pBac vector with AflI, ApaI, NheI, StuI, XhoI overhangs
JURv_Sv40	TCACTCGAGGCTAGCAGGCCTGATGAGTTTGGACAAA CACAACT	PCR amplification of pBac vector with AflI, ApaI, NheI, StuI, XhoI overhangs

JUFwtubulin	CATCTCGAGACCTCACACTTGCCGTAAATGGAG	Cloning of *Tc-αTubulin1* with XhoI overhang
JURvtubulin	GTATCGCTAGCTTGGTAGTTGAGTTTTACAAATTAC	Cloning of *Tc-αTubulin1* with NheI overhang
JUFw_IR	GAGTCTCTGCACTGAACATTGT	Sequencing of IR vector
JURv_IR	TCACTGCATTCTAGTTGTGGT	Sequencing of IR vector
JUFw_IR2	CGGTTATGATGGTGCAACGA	Sequencing of IR vector
JURv_IR2	CATCATAACCGTTCACTGCAG	Sequencing of IR vector
JUFW_pSL	CGACTTTAACAAAGTTGAGTGAATTA	Sequencing of VSRs in pSLfa vector
JURv_pSL	GTGGTATGGCTGATTATGATCTA	Sequencing of VSRs in pSLfa vector
JuFw_pBac	GCTGGCTTCGGTTTATATGAGA	Sequencing of VSRs in pBac vector
JURv_pBac	GAGTCTCTGCACTGAACATTGTCA	Sequencing of VSRs in pBac vector

7.3 Identification of novel RNAi target genes in *Tribolium*

Tab. 7.4: Top 100 RNAi target genes. The gene symbols, the iB numbers, the *Tribolium* genes and the orthologous *Drosophila* genes are shown.

Symbol	iB	Tc gene	Dm gene	Dm gene name
control	iB_05139	TC011976	CG3331	ebony
L1	iB_00003	TC000029	CG1212	p130CAS
L2	iB_00004	TC000040	CG42341	Pka-R1
L3	iB_00011	TC000069	CG4097	Prosβ6
L4	iB_00015	TC000082	CG7506	-
L5	iB_00029	TC000161	CG6202	Surf4
L6	iB_00031	TC000165	CG40470	-
L7	iB_00053	TC000258	CG4904	Prosα6
L8	iB_00063	TC032227	CG5706	-
L9	iB_00141	TC000614	CG18495	Prosα1
L10	iB_00322	TC002003	CG5848	cact
L11	iB_00404	TC002574	CG4659	Srp54k
L12	iB_00411	TC002601	CG1743	Gs2
L13	iB_00462	TC002881	CG11856	Nup358
L14	iB_00472	TC002933	CG8749	snRNP-U1-70K
L15	iB_00503	TC003020	CG14813	δCOP-PB
L16	iB_00512	TC003079	CG15609	Ehbp1-PG
L17	iB_00606	TC003747	CG2063	-
L18	iB_00607	TC003752	CG18412	ph-p
L19	iB_00621	TC003818	CG9854	hrg
L20	iB_00655	TC004029	CG4153	eIF-2β
L21	iB_00731	TC004599	CG7007	VhaPPA1
L22	iB_00753	TC004683	CG8843	Sec5
L23	iB_00788	TC004949	CG18617	Vha100-2
L24	iB_00825	TC005313	CG5395	nmd
L25	iB_00950	TC005869	CG18174	Rpn11
L26	iB_00985	TC006047	CG5269	vib
L27	iB_01026	TC006281	CG17332	VhaSFD
L28	iB_01103	TC006982	-	-

L29	iB_01207	TC007510	CG9177	eIF5
L30	iB_01233	TC007640	CG7957	MED17
L31	iB_01271	TC007846	CG8432	Rep
L32	iB_01280	TC007891	CG4157	Rpn12
L33	iB_01347	TC008407	CG31004	mesh
L34	iB_01385	TC008671	CG10938	Prosα5
L35	iB_01456	TC009010	CG7610	ATPsyn-γ
L36	iB_01467	TC033679	CG18076	shot
L37	iB_01493	TC009191	CG1250	Sec23
L38	iB_01528	TC009328	CG10540	cpa
L39	iB_01562	TC009491	CG2910	nito
L40	iB_01675	TC010367	CG1088	Vha26
L41	iB_01682	TC010405	CG3992	srp
L42	iB_01709	TC010557	CG9539	Sec61α
L43	iB_01800	TC011082	CG2674	Sam-S
L44	iB_01807	TC011120	CG15811	Rop
L45	iB_01824	TC011204	CG17420	RpL15
L46	iB_01879	TC011708	CG5930	TfIIA-L
L47	iB_02161	TC013571	CG6625	alpha snap
L48	iB_03390	TC002507	CG17603	Taf1
L49	iB_00268	TC001600	CG6601	Rab6
L50	iB_01793	TC011058	CG18102	shi
L51	iB_01796	TC011068	CG2467	pot
L52	iB_01857	TC031473	CG1782	Uba1
L53	iB_01884	TC011725	CG6022	Cchl
L54	iB_02377	TC015014	CG9012	Chc
L55	iB_02456	TC015321	CG6593	pp1alpha-96a
L56	iB_02457	TC015322	CG11154	ATPsyn-β
L57	iB_02509	TC015727	CG17170	su(f)
L58	iB_02531	TC015935	CG13281	Cas
L59	iB_02553	TC031972	CG5931	l(3)72Ab
L60	iB_02593	TC030579	CG6174	Arp1
L61	iB_02601	TC030625	CG12323	Prosβ5
L62	iB_02611	TC030666	CG11522	RpL6
L63	iB_02644	TC000801	CG2503	atms
L64	iB_02646	TC030619	CG8118	mam
L65	iB_02695	TC003405	-	-
L66	iB_02765	TC008190	CG12202	Nat1
L67	iB_02771	TC008263	CG10228	inr-a
L68	iB_02788	TC013746	-	-
L69	iB_02796	TC014300	CG18398	Tango6
L70	iB_02797	TC031145	CG34418	sif
L71	iB_02808	TC033251	-	-
L72	iB_02921	TC012455	CG9206	Gl
L73	iB_03608	TC003731	CG3780	Spx
L74	iB_03656	TC003944	-	-
L75	iB_03752	TC004420	CG1703	-
L76	iB_03754	TC004425	CG4147	hsc70-3
L77	iB_03836	TC004760	CG11152	fd102C
L78	iB_04084	TC006188	CG4644	mtRNApol
L79	iB_04099	TC006253	CG8566	unc-104
L80	iB_04125	TC006375	CG5378	Rpn7
L81	iB_04162	TC006514	CG5341	Sec6
L82	iB_04192	TC006679	CG31992	gw

L83	iB_04235	TC006902	-	-
L84	iB_04411	TC007999	CG16916	Rpt3
L85	iB_04531	TC008662	CG4584	dUTPase
L86	iB_04581	TC009040	CG4548	XNP
L87	iB_05229	TC012381	CG6843	-
L88	iB_05303	TC012723	CG12104	-
L89	iB_05370	TC013003	CG5971	Cdc6
L90	iB_05404	TC013245	CG10002	fkh
L91	iB_05526	TC013939	CG1109	-
L92	iB_05560	TC014109	CG31155	Rpb7
L93	iB_05604	TC014294	CG1828	dre4
L94	iB_05628	TC014413	CG7269	Hel25E
L95	iB_05683	TC014730	CG12245	gcm
L96	iB_05693	TC014785	CG12750	ncm
L97	iB_05726	TC014931	CG2909	-
L98	iB_05983	TC016270	CG3962	Keap1
L99	iB_06042	TC032389	CG5081	Syx7
L100	iB_06050	TC030708	CG2252	fs(1)h

Tab. 7.5: Top 40 RNAi target genes. The gene symbols, the iB numbers, the *Tribolium* genes and the orthologous *Drosophila* genes are shown.

Symbol	**iB**	**Tc gene**	**Dm gene**	**Dm gene name**
control	iB_05139	TC011976	CG3331	ebony
L2	iB_00004	TC000040	CG42341	Pka-R1
L3	iB_00011	TC000069	CG4097	Prosβ6
L5	iB_00029	TC000161	CG6202	Surf4
L7	iB_00053	TC000258	CG4904	Prosα6
L9	iB_00141	TC000614	CG18495	Prosα1
L10	iB_00322	TC002003	CG5848	cact
L11	iB_00404	TC002574	CG4659	Srp54k
L14	iB_00472	TC002933	CG8749	snRNP-U1-70K
L17	iB_00606	TC003747	CG2063	-
L25	iB_00950	TC005869	CG18174	Rpn11
L27	iB_01026	TC006281	CG17332	VhaSFD
L32	iB_01280	TC007891	CG4157	Rpn12
L37	iB_01493	TC009191	CG1250	Sec23
L39	iB_01562	TC009491	CG2910	nito
L42	iB_01709	TC010557	CG9539	Sec61α
L43	iB_01800	TC011082	CG2674	Sam-S
L44	iB_01807	TC011120	CG15811	Rop
L47	iB_02161	TC013571	CG6625	alpha snap
L50	iB_01793	TC011058	CG18102	shi
L52	iB_01857	TC031473	CG1782	Uba1
L54	iB_02377	TC015014	CG9012	Chc
L55	iB_02456	TC015321	CG6593	pp1alpha-96a
L56	iB_02457	TC015322	CG11154	ATPsyn-β
L58	iB_02531	TC015935	CG13281	Cas
L61	iB_02601	TC030625	CG12323	Prosβ5
L62	iB_02611	TC030666	CG11522	RpL6
L63	iB_02644	TC000801	CG2503	atms

L64	iB_02646	TC030619	CG8118	mam
L67	iB_02771	TC008263	CG10228	inr-a
L76	iB_03754	TC004425	CG4147	hsc70-3
L79	iB_04099	TC006253	CG8566	unc-104
L80	iB_04125	TC006375	CG5378	Rpn7
L82	iB_04192	TC006679	CG31992	gw
L84	iB_04411	TC007999	CG16916	Rpt3
L88	iB_05303	TC012723	CG12104	-
L90	iB_05404	TC013245	CG10002	fkh
L93	iB_05604	TC014294	CG1828	dre4
L94	iB_05628	TC014413	CG7269	Hel25E
L95	iB_05683	TC014730	CG12245	gcm
L100	iB_06050	TC030708	CG2252	fs(1)h

Tab. 7.6: Lethal genes found by GO term combinations of cluster 1, 2 and 7. By searching for genes that share GO term combinations of cluster 1, 2 and 7 (see Fig. 4.6 C and Fig. 7.3 for clusters) additional RNAi target genes (given are the iB numbers) were found which showed strong lethality (70-100%) in the *iBeetle* screen after larval or pupal RNAi.

Cluster 1 [iB_genes]	**iB_pupal lethality in %**	**iB_larval lethality in %**
iB_01385	100.0	90.0
iB_05343	60.0	90.0
iB_04808	100.0	100.0
iB_04154	100.0	100.0
iB_00780	100.0	100.0
iB_01375	100.0	100.0
Cluster 2 [iB_genes]	**iB_pupal lethality in %**	**iB_larval lethality in %**
iB_01385	100.0	90.0
iB_00780	100.0	100.0
iB_01375	100.0	100.0
Cluster 7 [iB_genes]	**iB_pupal lethality in %**	**iB_larval lethality in %**
iB_02921	100.0	80.0
iB_02249	80.0	100.0
iB_00560	90.0	100.0
iB_06329	100.0	100.0
iB_04169	60.0	100.0
iB_03627	80.0	100.0
iB_00655	100.0	100.0
iB_00476	90.0	90.0
iB_01431	100.0	100.0
iB_00704	50.0	100.0
iB_05580	80.0	100.0
iB_02653	40.0	100.0
iB_00929	90.0	100.0
iB_01820	100.0	100.0
iB_01531	100.0	100.0
iB_01977	60.0	70.0
iB_01181	60.0	88.89
iB_03608	90.0	100.0
iB_06425	100.0	20.0
iB_05731	70.0	90.0
iB_02215	80.0	50.0

iB_01311	100.0	20.0
iB_06301	90.0	0.0
iB_01484	70.0	100.0
iB_00920	60.0	80.0
iB_05243	90.0	70.0
iB_05446	80.0	88.89
iB_01552	70.0	100.0
iB_01666	70.0	100.0
iB_01166	90.0	100.0
iB_01965	90.0	100.0
iB_01215	80.0	90.0
iB_05428	90.0	80.0
iB_00780	100.0	100.0
iB_04868	70.0	60.0
iB_01375	100.0	100.0
iB_02877	80.0	80.0

7.4 Single and double RNAi treatments of RNAi target genes

Tab. 7.7: RNAi of non-overlapping dsRNA fragments. Lethality was confirmed by injection of non-overlapping dsRNAis (1µg/µl). Injections were done into 10 larvae, respectively, and survival animals were counted every second day post injection. *Tc-ebony* was used as negative control. d= day

	d2	**d4**	**d6**	**d8**	**d10**
Tc-ebony 1µg/µl	10	10	9	9	9
L2_2 1µg/µl	6	2	2	0	0
L3_2 1µg/µl	9	4	0	0	0
L5_2 1µg/µl	8	0	0	0	0
L7_2 1µg/µl	9	6	0	0	0
L9_2 1µg/µl	7	6	0	0	0
L10_2 1µg/µl	6	0	0	0	0
L11_2 1µg/µl	10	1	0	0	0
L14_2 1µg/µl	6	5	3	0	0
L17_2 1µg/µl	7	3	2	0	0
L25_2 1µg/µl	8	5	0	0	0
L27_2 1µg/µl	10	5	0	0	0
L30_2 1µg/µl	7	4	2	0	0
L32_2 1µg/µl	7	4	4	0	0
L37_2 1µg/µl	9	6	1	0	0
L39_2 1µg/µl	10	4	2	0	0
L42_2 1µg/µl	7	4	1	0	0
L43_2 1µg/µl	10	7	2	0	0
L44_2 1µg/µl	7	1	0	0	0
L47_2 1µg/µl	10	2	0	0	0
L50_2 1µg/µl	10	2	0	0	0
L52_2 1µg/µl	8	4	0	0	0
L54_2 1µg/µl	9	3	0	0	0
L55_2 1µg/µl	10	4	0	0	0
L56_2 1µg/µl	10	3	0	0	0
L58_2 1µg/µl	10	6	5	0	0

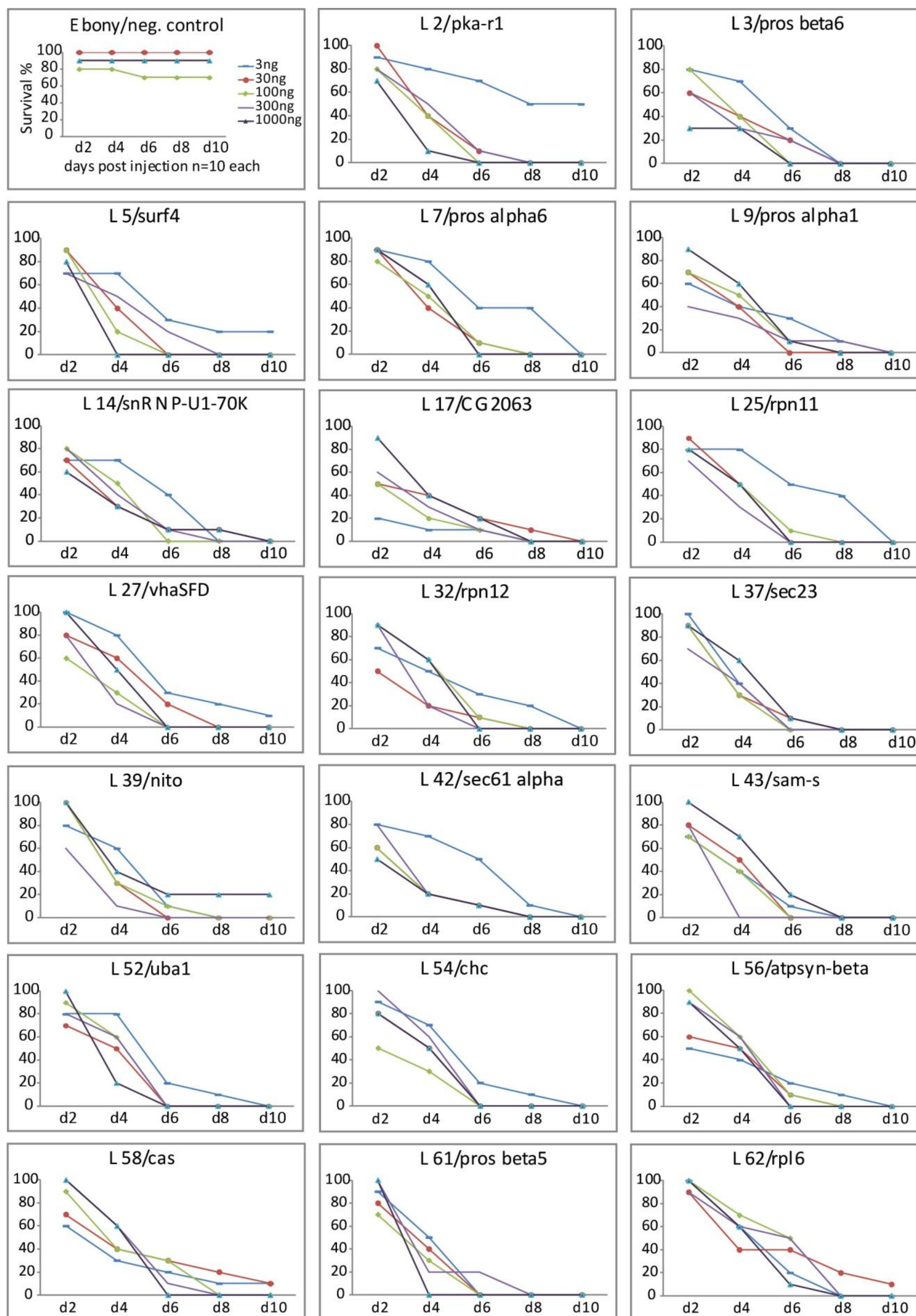
L61_2 1µg/µl	10	3	0	0	0
L61_3 1µg/µl	9	2	0	0	0
L62_2 1µg/µl	9	3	1	0	0
L62_3 1µg/µl	10	3	0	0	0
L63_2 1µg/µl	10	9	5	0	0
L64_2 1µg/µl	10	9	8	0	0
L67_2 1µg/µl	8	0	0	0	0
L76_2 1µg/µl	10	5	0	0	0
L79_2 1µg/µl	10	2	0	0	0
L80_2 1µg/µl	10	0	0	0	0
L82_2 1µg/µl	10	1	0	0	0
L84_2 1µg/µl	10	1	0	0	0
L88_2 1µg/µl	10	9	5	2	0
L90_2 1µg/µl	10	6	1	0	0
L90_3 1µg/µl	10	7	0	0	0
L93_2 1µg/µl	7	1	0	0	0
L94_2 1µg/µl	10	1	0	0	0
L95_2 1µg/µl	10	0	0	0	0
L100_2 1µg/µl	10	5	0	0	0

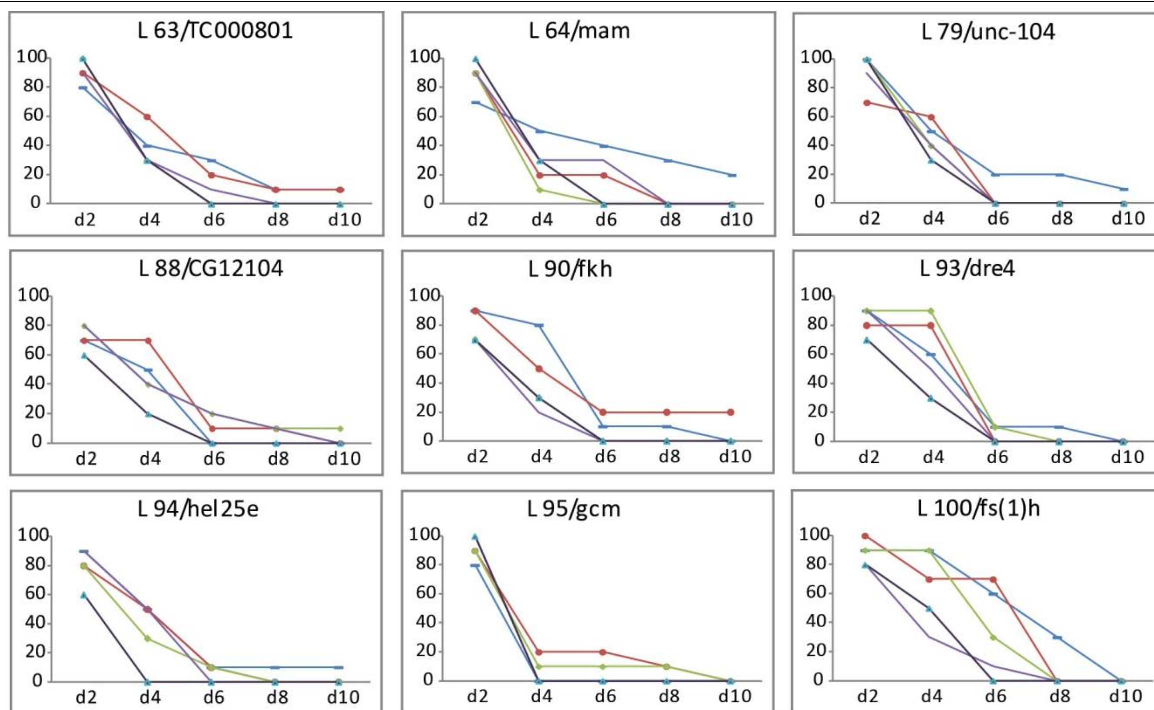
Tab. 7.8: Single and double RNAi treatments. RNAis were performed in 10 penultimate instar larvae (L6), respectively. Each total dsRNA concentration was 0.5ng/µl. Number of surviving animals was documented every second day post injection and compared to the single injections. d=day

	d2	d4	d6	d8	d10
L11	9	9	7	7	5
L11+10	10	8	8	6	2
L11+44	10	10	9	8	4
L11+47	10	8	8	6	3
L11+50	10	9	5	2	2
L11+55	10	10	10	9	8
L11+67	10	7	5	4	2
L11+76	10	10	10	9	2
L11+80	10	9	8	7	5
L11+82	10	10	9	4	3
L11+84	10	10	10	7	2
L50	10	9	9	7	5
L50+10	10	8	6	5	5
L50+44	10	10	10	10	3
L50+47	10	8	5	2	0
L50+55	8	7	7	4	3
L50+67	10	9	8	6	3
L50+76	10	10	7	5	3
L50+80	9	9	9	7	5
L50+82	10	10	6	6	4
L50+84	8	8	7	5	3
L67	10	8	6	4	0
L67+10	10	10	5	5	4
L67+44	10	9	9	9	4
L67+47	10	8	7	3	1
L67+55	10	9	5	4	3
L67+76	10	6	5	3	1
L67+80	9	9	9	9	7

L67+82	10	8	6	2	0
L67+84	10	8	7	5	3
L76	10	9	5	1	0
L76+10	9	8	8	5	3
L76+44	10	5	5	4	4
L76+47	9	6	5	3	0
L76+55	10	9	7	7	4
L76+80	10	9	8	5	3
L76+82	10	9	9	6	3
L76+84	10	10	10	6	4
L84	10	9	6	3	1
L84+10	10	10	10	7	7
L84+44	10	9	9	8	3
L84+47	10	5	5	3	0
L84+55	10	10	10	9	7
L84+80	9	7	7	7	3
L84+82	10	7	7	3	2
L55	10	10	10	10	8
L55+10	10	10	9	6	7
L55+44	9	8	8	3	2
L55+47	10	10	6	4	1
L55+80	10	10	10	9	8
L55+82	9	9	9	4	1
L80	9	6	5	2	1
L80+10	10	8	8	7	6
L80+44	9	8	8	6	2
L80+47	10	10	9	7	1
L80+82	10	9	8	7	2
L82	10	8	7	2	0
L82+10	10	8	7	5	3
L82+44	10	9	9	5	2
L82+47	10	7	7	2	0
L44	9	8	8	7	0
L44+10	10	8	8	6	4
L44+47	10	8	6	3	0
L47	10	6	5	3	1
L47+10	10	4	4	2	1
L10	10	5	2	2	0
Tc-ebony	10	9	9	9	9

Fig. 7.1: Identification of 40 novel RNAi target genes in *Tribolium castaneum*

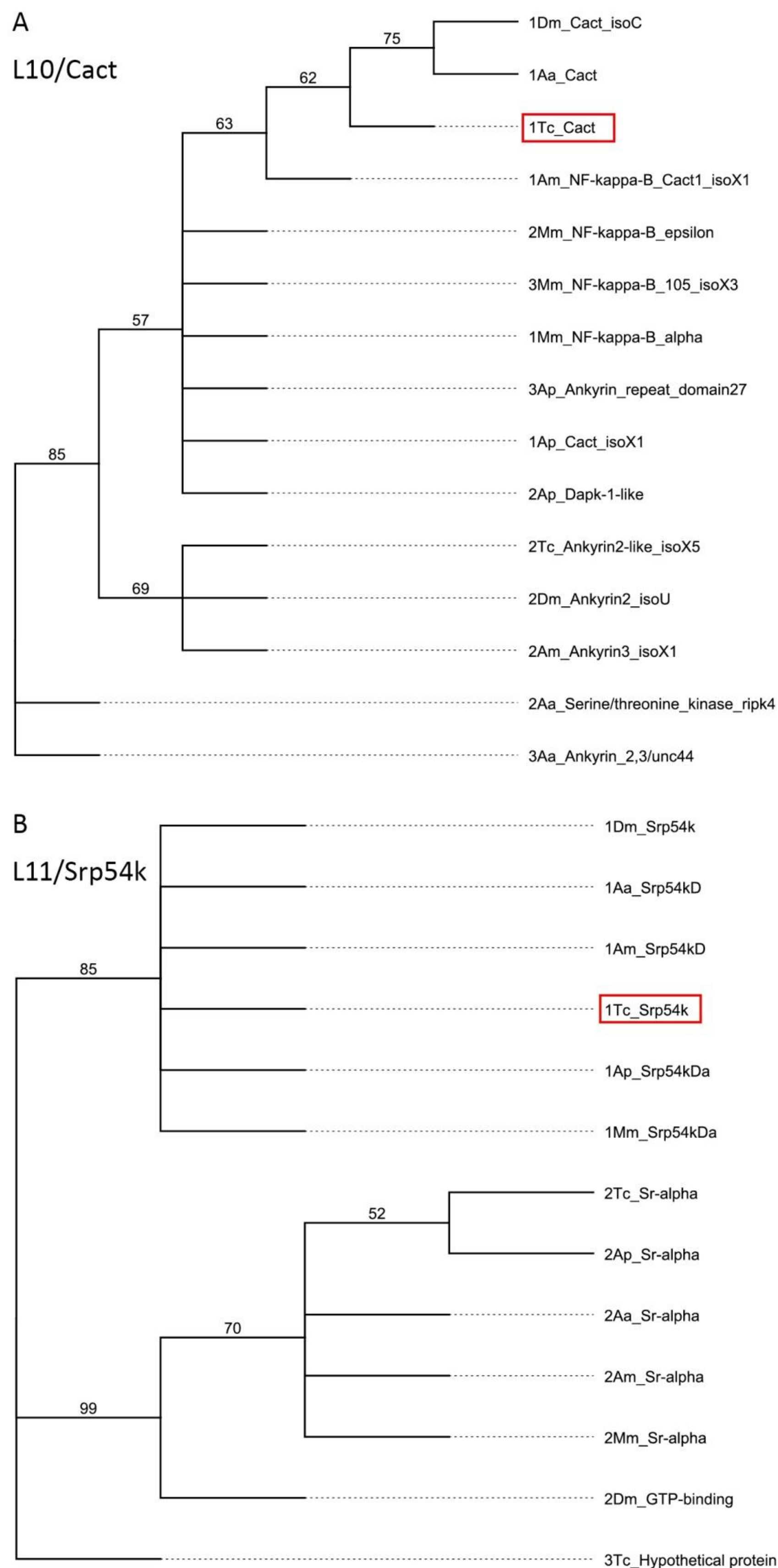


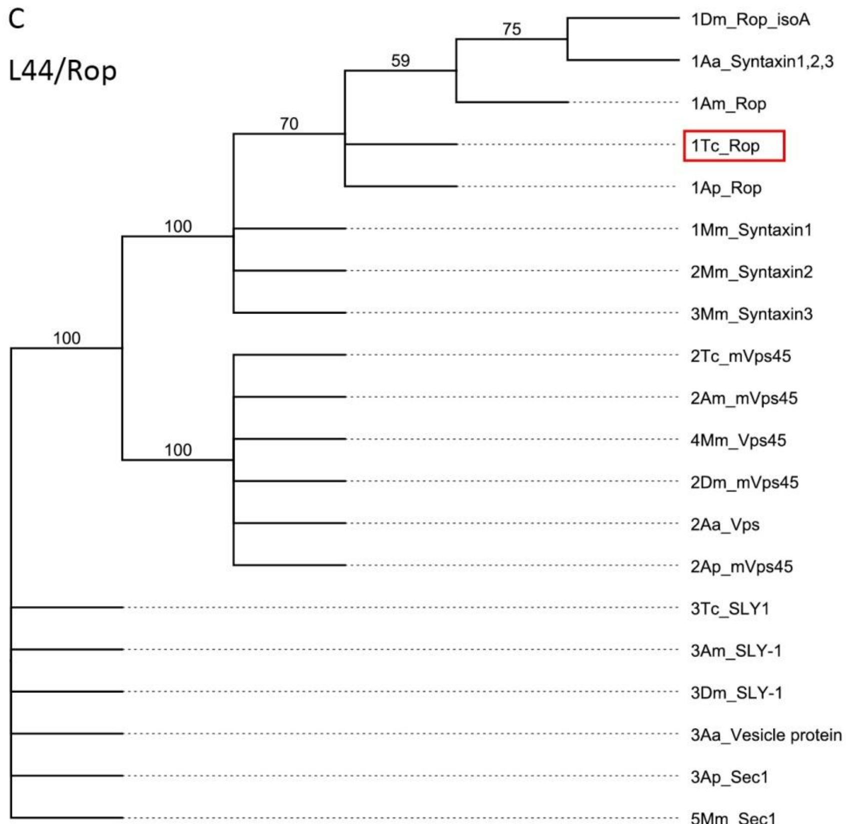


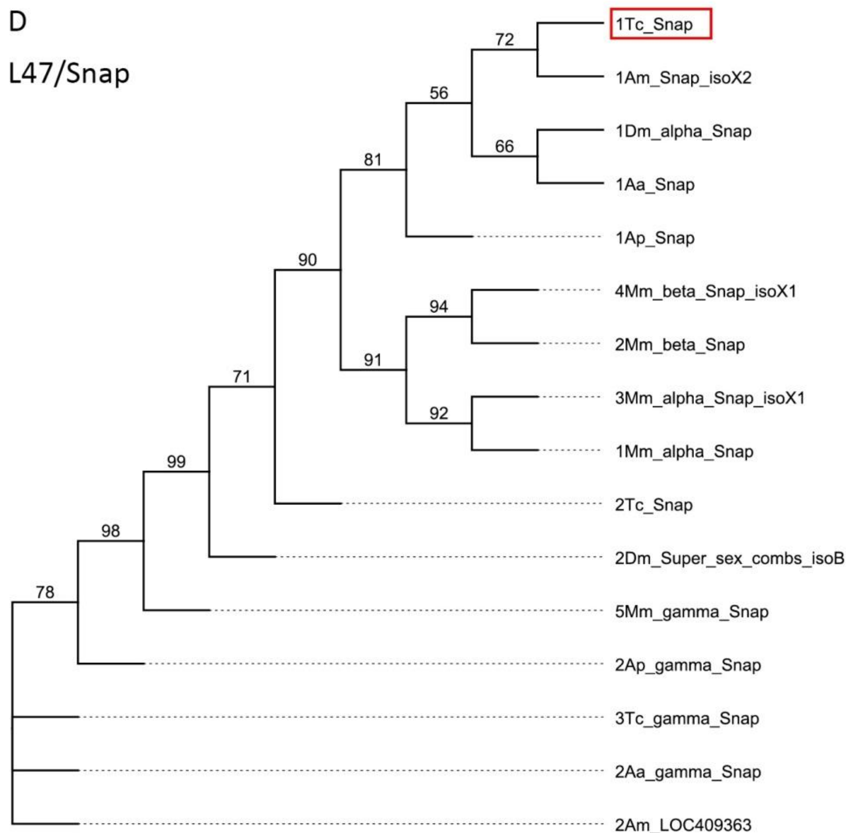
Based on data of the *iBeetle* screen, the 100 most efficient RNAi target genes were selected and retested using different dsRNA concentrations. The results for the most efficient 40 lethal genes are shown. The corresponding results of the top eleven candidates are shown in Fig. 4.1. See further details in figure legend of Fig. 4.1.

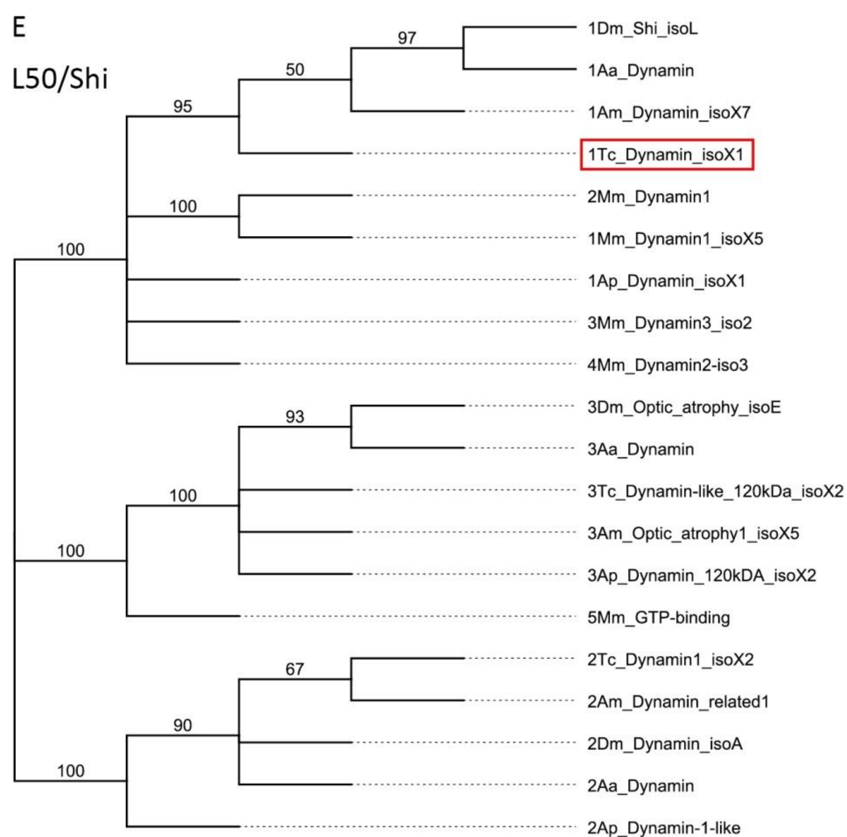
7.5 Phylogeny and GO term analysis

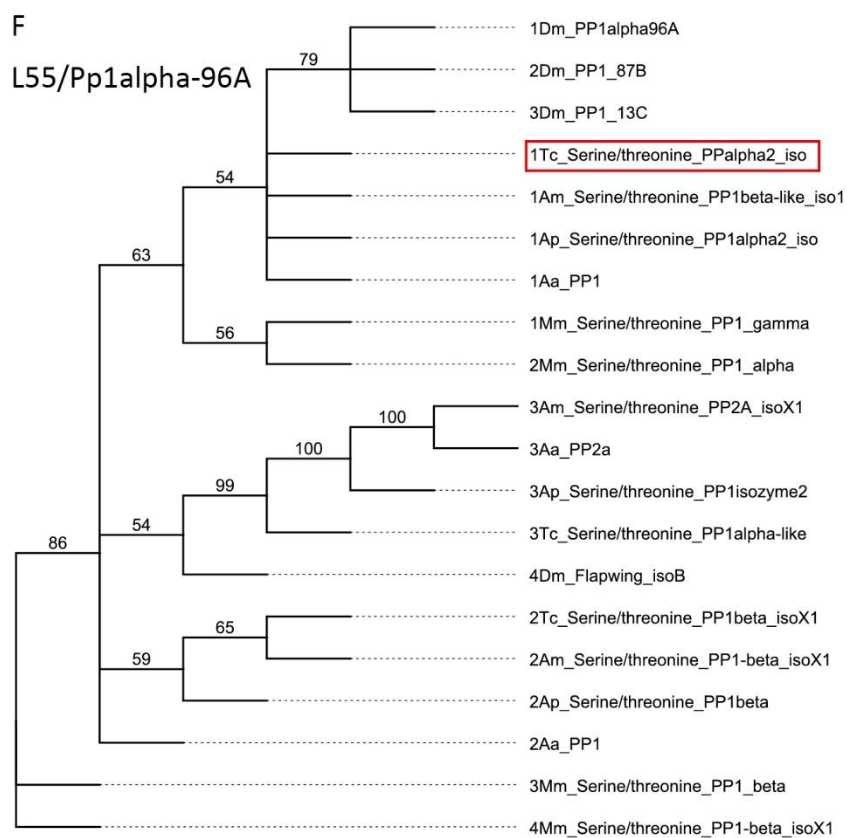
Fig. 7.2: Phylogenetic trees of the novel RNAi target genes




C
L44/Rop
75
59
70
100
100
100
1Dm_Rop_isoA
1Aa_Syntaxin1,2,3
1Am_Rop
1Tc_Rop
1Ap_Rop
1Mm_Syntaxin1
2Mm_Syntaxin2
3Mm_Syntaxin3
2Tc_mVps45
2Am_mVps45
4Mm_Vps45
2Dm_mVps45
2Aa_Vps
2Ap_mVps45
3Tc_SLY1
3Am_SLY-1
3Dm_SLY-1
3Aa_Vesicle protein
3Ap_Sec1
5Mm_Sec1

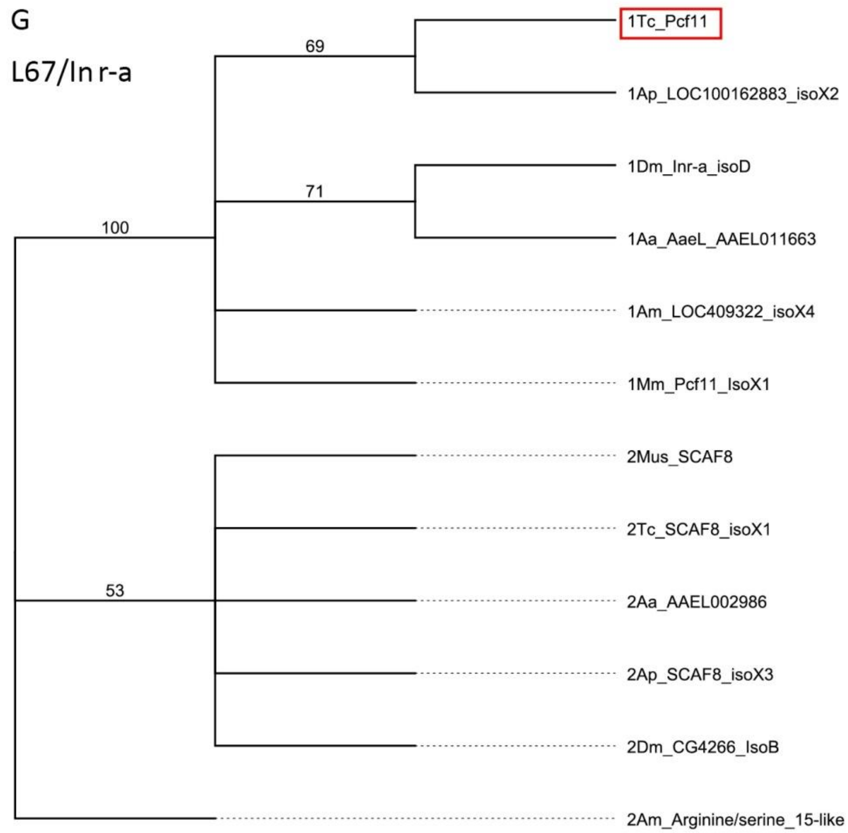

D
L47/Snap
72
56
66
81
90
94
91
92
71
99
98
78
1Tc_Snap
1Am_Snap_isoX2
1Dm_alpha_Snap
1Aa_Snap
1Ap_Snap
4Mm_beta_Snap_isoX1
2Mm_beta_Snap
3Mm_alpha_Snap_isoX1
1Mm_alpha_Snap
2Tc_Snap
2Dm_Super_sex_combs_isoB
5Mm_gamma_Snap
2Ap_gamma_Snap
3Tc_gamma_Snap
2Aa_gamma_Snap
2Am_LOC409363


E
L50/Shi
97
50
95
100
100
93
100
100
67
90
100
1Dm_Shi_isoL
1Aa_Dynamin
1Am_Dynamin_isoX7
1Tc_Dynamin_isoX1
2Mm_Dynamin1
1Mm_Dynamin1_isoX5
1Ap_Dynamin_isoX1
3Mm_Dynamin3_iso2
4Mm_Dynamin2-iso3
3Dm_Optic_atrophy_isoE
3Aa_Dynamin
3Tc_Dynamin-like_120kDa_isoX2
3Am_Optic_atrophy1_isoX5
3Ap_Dynamin_120kDA_isoX2
5Mm_GTP-binding
2Tc_Dynamin1_isoX2
2Am_Dynamin_related1
2Dm_Dynamin_isoA
2Aa_Dynamin
2Ap_Dynamin-1-like


F
L55/Pp1alpha-96A
79
54
63
56
100
100
99
54
86
65
59
1Dm_PP1alpha96A
2Dm_PP1_87B
3Dm_PP1_13C
1Tc_Serine/threonine_PPalpha2_iso
1Am_Serine/threonine_PP1beta-like_iso1
1Ap_Serine/threonine_PP1alpha2_iso
1Aa_PP1
1Mm_Serine/threonine_PP1_gamma
2Mm_Serine/threonine_PP1_alpha
3Am_Serine/threonine_PP2A_isoX1
3Aa_PP2a
3Ap_Serine/threonine_PP1isozyme2
3Tc_Serine/threonine_PP1alpha-like
4Dm_Flapwing_isoB
2Tc_Serine/threonine_PP1beta_isoX1
2Am_Serine/threonine_PP1-beta_isoX1
2Ap_Serine/threonine_PP1beta
2Aa_PP1
3Mm_Serine/threonine_PP1_beta
4Mm_Serine/threonine_PP1-beta_isoX1

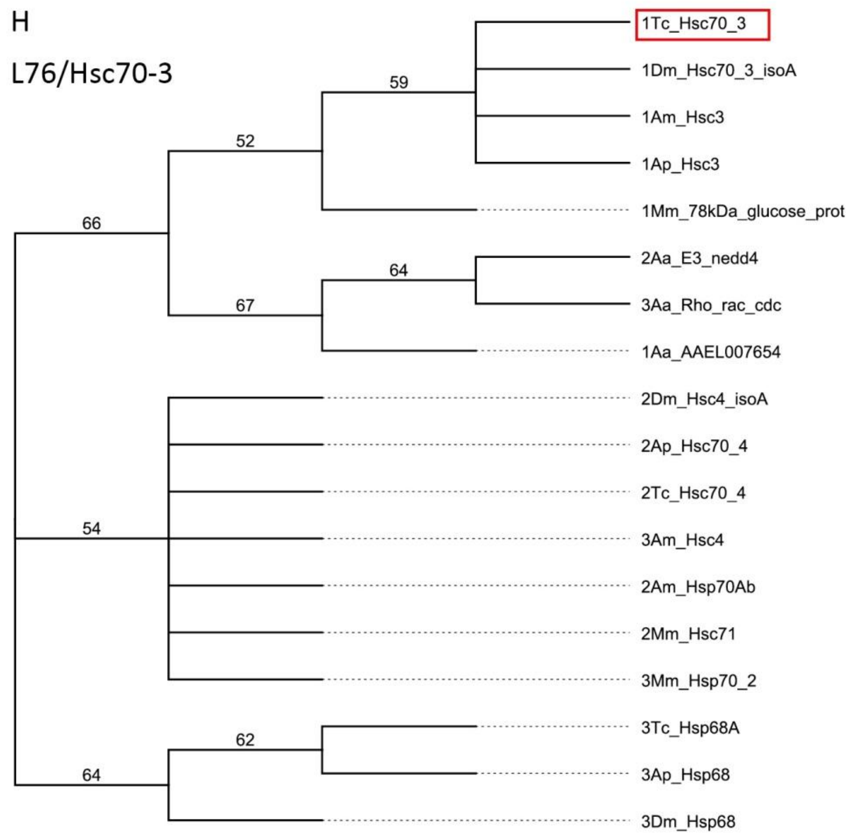
G

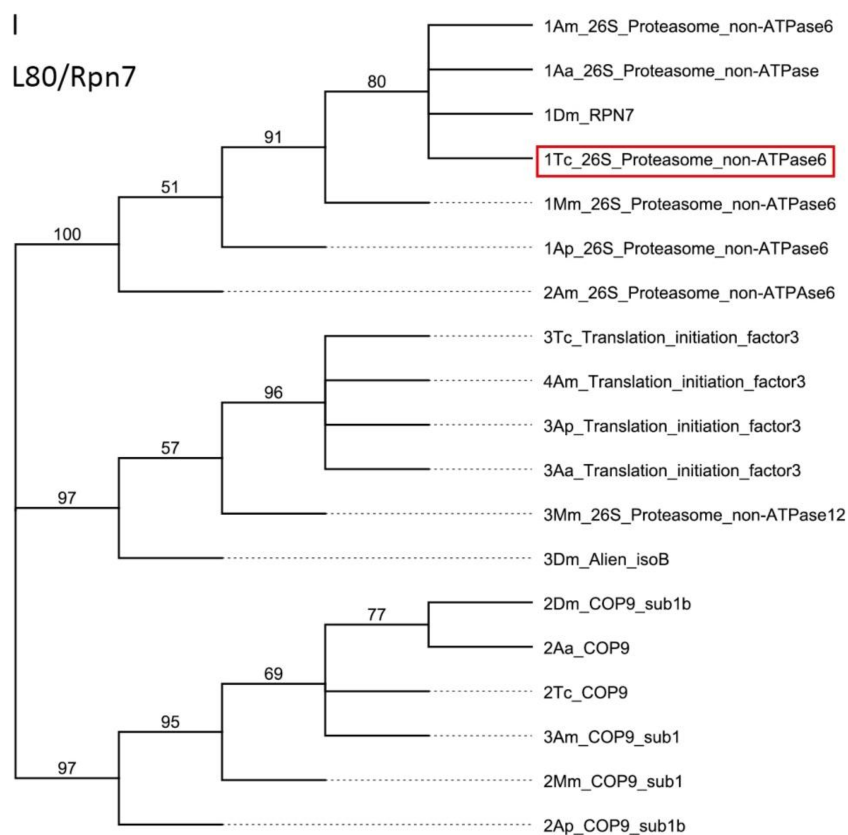
L67/In r-a

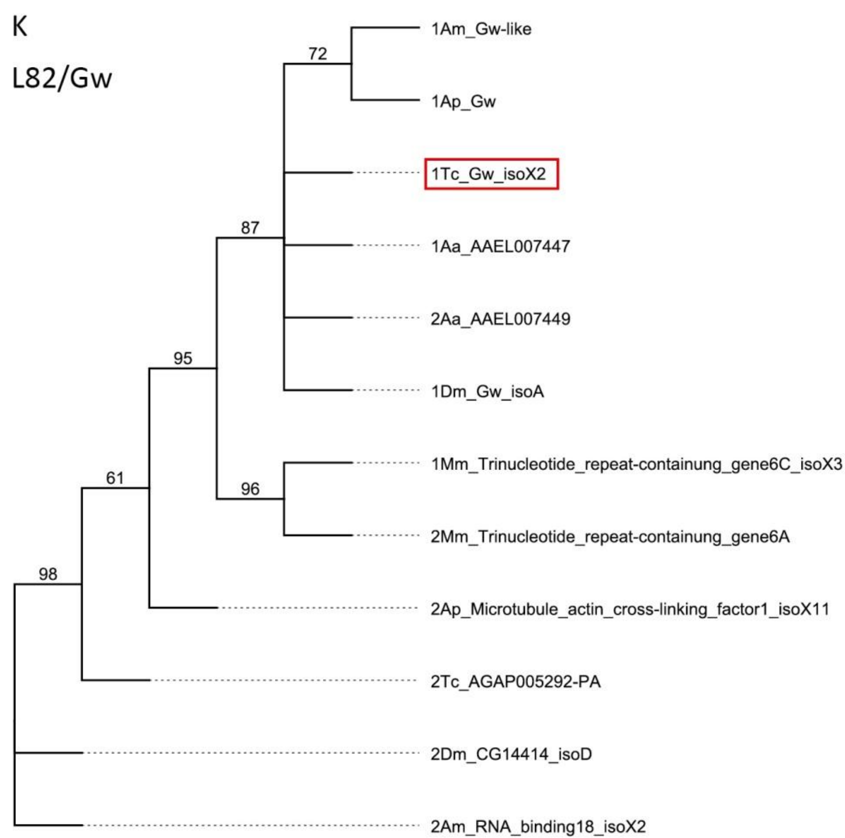


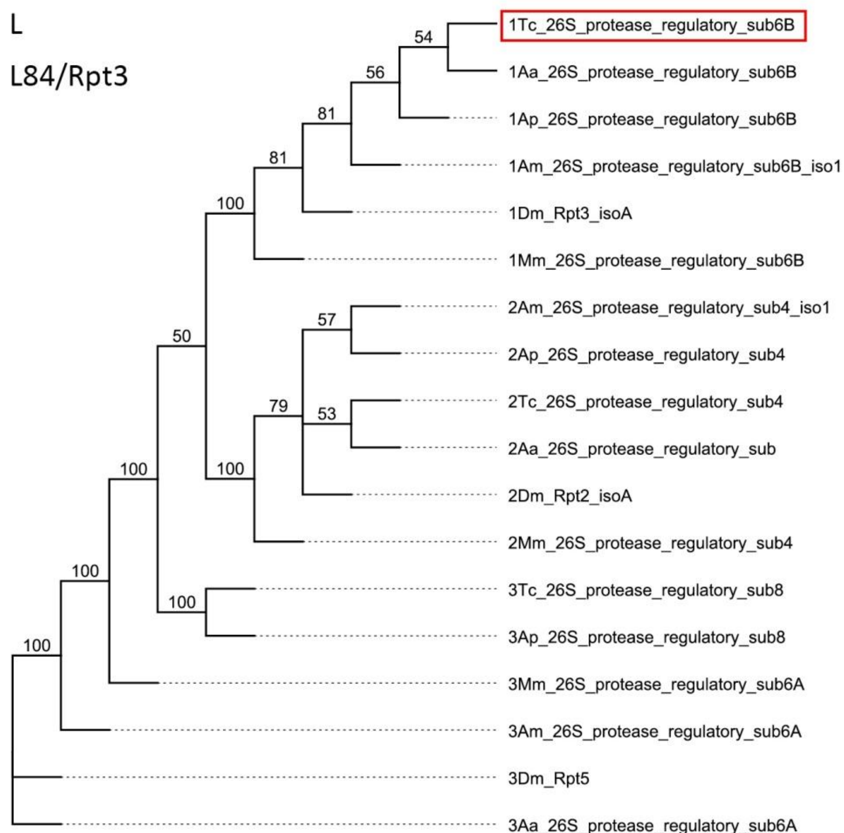
H

L76/Hsc70-3




I
L80/Rpn7
1Am_26S_Proteasome_non-ATPase6
1Aa_26S_Proteasome_non-ATPase
1Dm_RPN7
1Tc_26S_Proteasome_non-ATPase6
1Mm_26S_Proteasome_non-ATPase6
1Ap_26S_Proteasome_non-ATPase6
2Am_26S_Proteasome_non-ATPAse6
3Tc_Translation_initiation_factor3
4Am_Translation_initiation_factor3
3Ap_Translation_initiation_factor3
3Aa_Translation_initiation_factor3
3Mm_26S_Proteasome_non-ATPase12
3Dm_Alien_isoB
2Dm_COP9_sub1b
2Aa_COP9
2Tc_COP9
3Am_COP9_sub1
2Mm_COP9_sub1
2Ap_COP9_sub1b
80
91
51
100
96
57
97
77
69
95
97


K
L82/Gw
1Am_Gw-like
1Ap_Gw
1Tc_Gw_isoX2
1Aa_AAEL007447
2Aa_AAEL007449
1Dm_Gw_isoA
1Mm_Trinucleotide_repeat-containung_gene6C_isoX3
2Mm_Trinucleotide_repeat-containung_gene6A
2Ap_Microtubule_actin_cross-linking_factor1_isoX11
2Tc_AGAP005292-PA
2Dm_CG14414_isoD
2Am_RNA_binding18_isoX2
72
87
95
61
96
98



(A-L) Phylogenetic trees were generated by ClustalW alignment as implemented in Geneious version 5.6.4 (Biomatters) by removing unclear parts of the sequences. Trees and images were created using Geneious Tree Builder and neighbour joining method. Dm *Drosophila melanogaster*, Am *Apis mellifera*, Aa *Aedes aegypti*, Ap *Acyrthosiphon pisum,* Mm *Mus musculus*.

Fig. 7.3: GO term analysis of top 40 RNAi target genes reveals additional targets

Annotation Cluster 1	Enrichment Score: 4,26			
Category	**Term**	**Count**	**%**	**PValue**
SP_PIR_KEYWORDS	proteasome	7	19	1,61E-09
GOTERM_CC_FAT	GO:0000502~proteasome complex	7	19	1,03E-06
KEGG_PATHWAY	dme03050:Proteasome	7	19	1,37E-06
GOTERM_MF_FAT	GO:0004175~endopeptidase activity	7	19	1,21E-02
GOTERM_BP_FAT	GO:0006508~proteolysis	8	22	2,39E-02
GOTERM_MF_FAT	GO:0070011~peptidase activity, acting on L-amino acid peptides	7	19	4,36E-02
Genes	CG16916, CG5378, CG18174, CG4157, CG4904, CG1782,CG4097, CG12323			
Annotation Cluster 2	**Enrichment Score: 2,37**			
SP_PIR_KEYWORDS	threonine protease	3	8	1,72E-03
INTERPRO	IPR001353:Proteasome, subunit alpha/beta	3	8	3,46E-03
GOTERM_MF_FAT	GO:0004298~threonine-type endopeptidase activity	3	8	5,05E-03
GOTERM_MF_FAT	GO:0070003~threonine-type peptidase activity	3	8	5,05E-03
GOTERM_CC_FAT	GO:0005839~proteasome core complex	3	8	9,76E-03
Genes	CG4904, CG4097, CG12323			
Annotation Cluster 3	**Enrichment Score: 2,22**			
GOTERM_BP_FAT	GO:0008104~protein localization	7	19	4,24E-03
GOTERM_BP_FAT	GO:0015031~protein transport	6	16	4,71E-03
GOTERM_BP_FAT	GO:0006886~intracellular protein transport	5	14	4,97E-03
GOTERM_BP_FAT	GO:0045184~establishment of protein localization	6	16	5,23E-03
GOTERM_BP_FAT	GO:0034613~cellular protein localization	5	14	5,49E-03
GOTERM_BP_FAT	GO:0070727~cellular macromolecule localization	5	14	1,59E-02
Genes	CG4659, CG15811, CG9539, CG6625, CG9012, CG5848, CG13281			
Annotation Cluster 4	**Enrichment Score: 2,10**			
GOTERM_BP_FAT	GO:0007268~synaptic transmission	5	14	6,65E-03
GOTERM_BP_FAT	GO:0019226~transmission of nerve impulse	5	14	7,55E-03
GOTERM_BP_FAT	GO:0007267~cell-cell signaling	5	14	9,74E-03
Genes	CG15811, CG18102, CG42341, CG6625, CG9012			
Annotation Cluster 5	**Enrichment Score: 2,09**			
GOTERM_BP_FAT	GO:0030163~protein catabolic process	6	16	1,12E-03
GOTERM_BP_FAT	GO:0044265~cellular macromolecule catabolic process	6	16	1,89E-03
GOTERM_BP_FAT	GO:0051603~proteolysis involved in cellular protein catabolic process	5	14	5,94E-03
GOTERM_BP_FAT	GO:0044257~cellular protein catabolic process	5	14	5,94E-03
GOTERM_BP_FAT	GO:0019941~modification-dependent protein catabolic process	4	11	2,99E-02
GOTERM_BP_FAT	GO:0043632~modification-dependent macromolecule catabolic process	4	11	3,04E-02
SP_PIR_KEYWORDS	Protease	4	11	3,37E-02
Genes	CG16916, CG18174, CG4904, CG1782, CG4097, CG12323, CG31992			
Annotation Cluster 6	**Enrichment Score: 2,01**			
SP_PIR_KEYWORDS	nucleotide-binding	9	24	6,56E-04
GOTERM_MF_FAT	GO:0032555~purine ribonucleotide binding	10	27	1,35E-02
GOTERM_MF_FAT	GO:0032553~ribonucleotide binding	10	27	1,35E-02
GOTERM_MF_FAT	GO:0017076~purine nucleotide binding	10	27	2,06E-02
GOTERM_MF_FAT	GO:0032559~adenyl ribonucleotide binding	8	22	3,71E-02
Genes	CG4659, CG16916, CG18102, CG4147, CG42341, CG11154, CG1782, CG7269, CG2674, CG8566			
Annotation Cluster 7	**Enrichment Score: 1,92**			
GOTERM_BP_FAT	GO:0000226~microtubule cytoskeleton organization	6	16	6,31E-03
GOTERM_BP_FAT	GO:0007052~mitotic spindle organization	5	14	7,28E-03
GOTERM_BP_FAT	GO:0007051~spindle organization	5	14	1,21E-02
GOTERM_BP_FAT	GO:0007010~cytoskeleton organization	6	16	3,67E-02
Genes	CG8749, CG42341, CG4157, CG12323, CG7269, CG11522			
Annotation Cluster 8	**Enrichment Score: 1,66**			
GOTERM_BP_FAT	GO:0043623~cellular protein complex assembly	4	11	4,40E-03
GOTERM_BP_FAT	GO:0034622~cellular macromolecular complex assembly	4	11	2,82E-02
GOTERM_BP_FAT	GO:0006461~protein complex assembly	4	11	2,90E-02
GOTERM_BP_FAT	GO:0070271~protein complex biogenesis	4	11	2,90E-02
GOTERM_BP_FAT	GO:0034621~cellular macromolecular complex subunit organization	4	11	4,75E-02
Genes	CG4659, CG15811, CG6625, CG13281			
Annotation Cluster 9	**Enrichment Score: 1,62**			
GOTERM_BP_FAT	GO:0010324~membrane invagination	5	14	1,92E-02
GOTERM_BP_FAT	GO:0006897~endocytosis	5	14	1,92E-02
GOTERM_BP_FAT	GO:0016044~membrane organization	5	14	3,80E-02
Genes	CG18102, CG6625, CG9012, CG5848, CG13281			
Annotation Cluster 10	**Enrichment Score: 1,56**			
GOTERM_BP_FAT	GO:0050657~nucleic acid transport	3	8	2,64E-02
GOTERM_BP_FAT	GO:0050658~RNA transport	3	8	2,64E-02
GOTERM_BP_FAT	GO:0051236~establishment of RNA localization	3	8	2,72E-02
GOTERM_BP_FAT	GO:0015931~nucleobase, nucleoside, nucleotide and nucleic acid transport	3	8	3,04E-02
Genes	CG17332, CG9012, CG7269			

The functional clusters as revealed by GO term analysis (DAVID database) using the top 40 RNAi target genes are displayed. GOTERM_BP_FAT where BP means biological process;

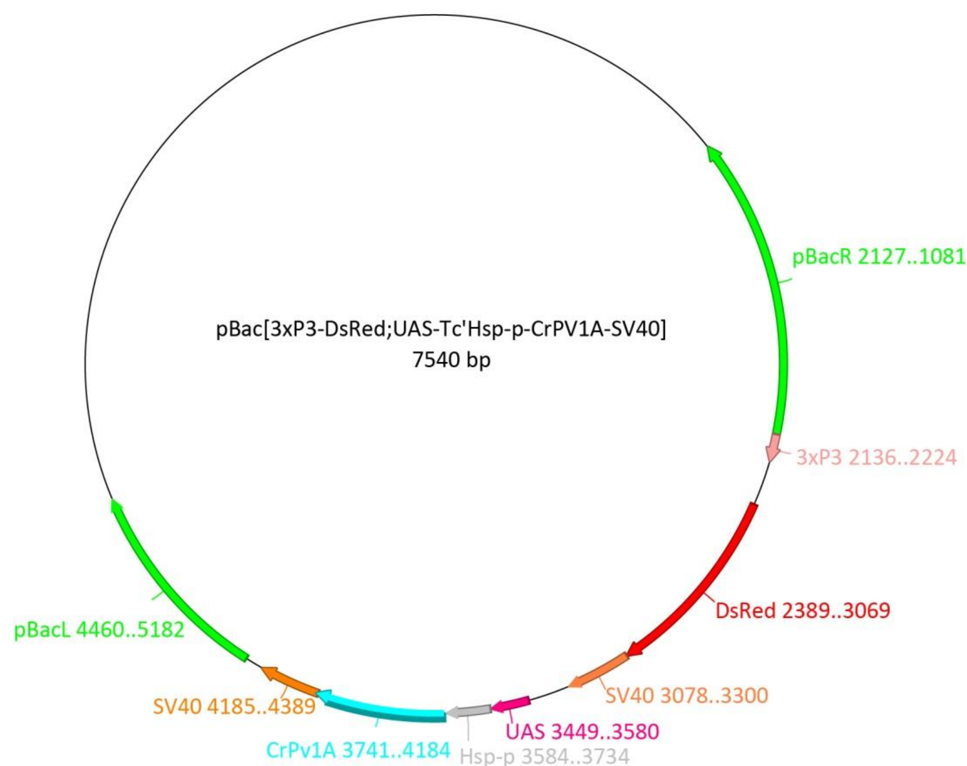
GOTERM_CC_FAT where CC means cellular component; GOTERM_MF_FAT where MF means molecular function; SP_PIR_KEYWORDS where PIR means protein information resource.

7.6 Repetition of *Tc-ebony* RNAi in transgenic inhibitor lines

Tab. 7.9: *Tc-ebony* knockdown in VSR lines. Repetition of *Tc-ebony* RNAi in transgenic lines carried Boje-Gal4 X UAS-VSRs and Dicer-i lines. Absolute numbers are given for each body color phenotype.

SB	**wt**	**dark brown**	**black**	**p38i^{55}**	**wt**	**dark brown**	**black**
n=48	6	14	28	n=18	0	6	13
CrPVi^{56}	**wt**	**dark brown**	**black**	**DCVi^{13}**	**wt**	**dark brown**	**black**
n=31	21	9	1	n=30	4	10	16
CrPVi^{61}	**wt**	**dark brown**	**black**	**DCVi^{21}**	**wt**	**dark brown**	**black**
n=16	12	4	0	n=28	1	6	21
B2i^{22}	**wt**	**dark brown**	**black**	**VP1i^{20}**	**wt**	**dark brown**	**black**
n=28	10	12	6	n=19	0	4	15
B2i^{40}	**wt**	**dark brown**	**black**	**VP1i^{23}**	**wt**	**dark brown**	**black**
n=15	2	3	10	n=23	3	5	15
p25i^{30}	**wt**	**dark brown**	**black**	**Dicer-i^{18}**	**wt**	**dark brown**	**black**
n=38	1	11	26	n=22	0	2	20
p25i^{35}	**wt**	**dark brown**	**black**	**Dicer-i^{22}**	**wt**	**dark brown**	**black**
n=26	0	10	16	n=25	0	9	16
p38i^{25}	**wt**	**dark brown**	**black**				
n=20	0	6	14				

7.7 Vector maps

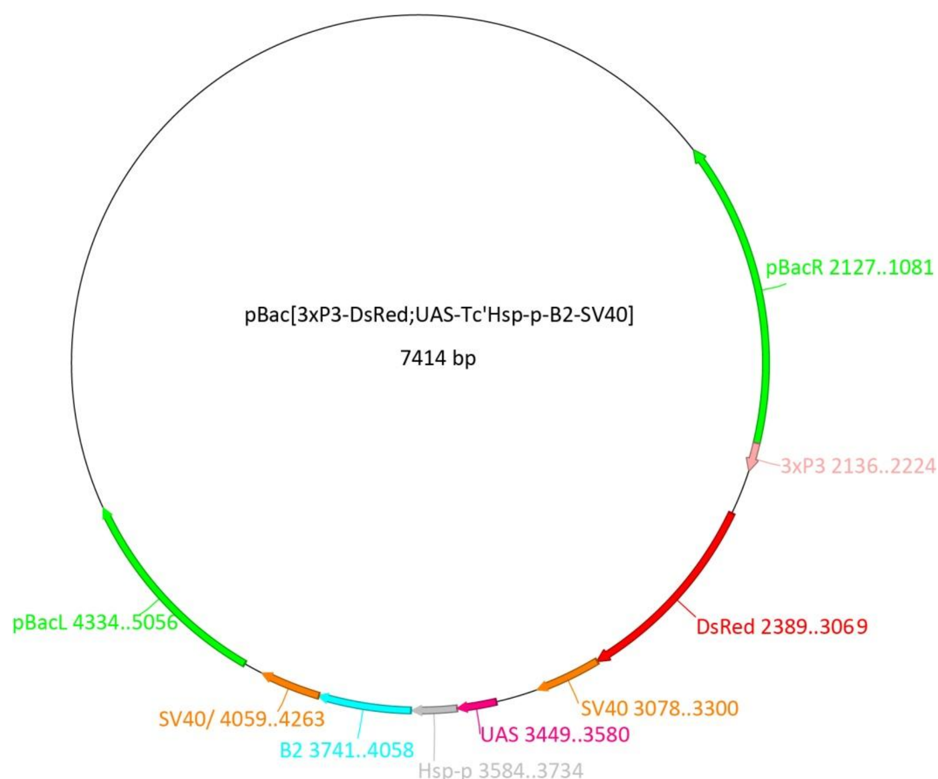


>pBac3xP3-DsRed;UAS-Tc'Hsp-p-CrPV1A-SV40

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGAT
GCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCA
GAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCG
CCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAG
GGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCC
AAGCTTTGTTTAAAATATAACAAAATTGTGATCCCACAAAATGAAGTGGGGCAAAATCAAATAATTAACTAGTGTCCGTAAAC
TTGTTGGTCTTCAACTTTTTGAGGAACACGTTGGACGGCAAATCGTGACTATAACACAAGTTGATTTAATAATTTTAGCCAAC
ACGTCGGGCTGCGTGTTTTTTGCGCTCTGTGTACACGTTGATTAACTGGTCGATTAAATAATTTAATTTTTGGTTCTTCTTTAA
ATCTGTGATGAAATTTTTTAAAATAACTTTAAATTCTTCATTGGTAAAAAATGCCACGTTTTGCAACTTGTGAGGGTCTAATAT
GAGGTCAAACTCAGTAGGAGTTTTATCCAAAAAAGAAAACATGATTACGTCTGTACACGAACGCGTATTAACGCAGAGTGCA
AAGTATAAGAGGGTTAAAAAATATATTTTACGCACCATATACGCATCGGGTTGATATCGTTAATATGGATCAATTTGAACAGT
TGATTAACGTGTCTCTGCTCAAGTCTTTGATCAAAACGCAAATCGACGAAAATGTGTCGGACAATATCAAGTCGATGAGCGA
AAAACTAAAAAGGCTAGAATACGACAATCTCACAGACAGCGTTGAGATATACGGTATTCACGACAGCAGGCTGAATAATAAA
AAAATTAGAAACTATTATTTAACCCTAGAAAGATAATCATATTGTGACGTACGTTAAAGATAATCATGCGTAAAATTGACGCAT
GTGTTTTATCGGTCTGTATATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTTATTATATTTACACTTACATACTAATA
ATAAATTCAACAAACAATTTATTTATGTTTATTTATTTATTAAAAAAAAACAAAAACTCAAAATTTCTTCTATAAAGTAACAAAAC
TTTTAAACATTCTCTCTTTTACAAAAATAAACTTATTTTGTACTTTAAAAACAGTCATGTTGTATTATAAAATAAGTAATTAGCTT
AACTTATACATAATAGAAACAAATTATACTTATTAGTCAGTCAGAAACAACTTTGGCACATATCAATATTATGCTCTCGACAAA
TAACTTTTTTGCATTTTTTGCACGATGCATTTGCCTTTCGCCTTATTTTAGAGGGGCAGTAAGTACAGTAAGTACGTTTTTTCA
TTACTGGCTCTTCAGTACTGTCATCTGATGTACCAGGCACTTCATTTGGCAAAATATTAGAGATATTATCGCGCAAATATCTC
TTCAAAGTAGGAGCTTCTAAACGCTTACGCATAAACGATGACGTCAGGCTCATGTAAAGGTTTCTCATAAATTTTTTGCGACT
TTGAACCTTTTCTCCCTTGCTACTGACATTATGGCTGTATATAATAAAAGAATTTATGCAGGCAATGTTTATCATTCCGTACAA
TAATGCCATAGGCCACCTATTCGTCTTCCTACTGCAGGTCATCACAGAACACATTTGGTCTAGCGTGTCCACTCCGCCTTTA
GTTTGATTATAATACATAACCATTTGCGGTTTACCGGTACTTTCGTTGATAGAAGCATCCTCATCACAAGATGATAATAAGTA
TACCATCTTAGCTGGCTTCGGTTTATATGAGACGAGAGTAAGGGGTCCGTCAAAACAAAACATCGATGTTCCCACTGGCCT
GGAGCGACTGTTTTTCAGTACTTCCGGTATCTCGCGTTTGTTTGATCGCACGGTTCCCACAATGCGGGGATTATTCATTAGA

GACTAATTCAATTAGAGCTAATTCAATTAGGATCCAAGCTTATCGATTTCGAACCCTCGACCGCCGGAGTATAAATAGAGGC
GCTTCGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTAAGCAAATAAACAAGC
GCAGCTGAACAAGCTAAACAATCGGGGTACCGCTAGAGTCGACGGTACCGCGGGCCCGGGATCCACCGGTCGCCACCAT
GGTGCGCTCCTCCAAGAACGTCATCAAGGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCACCGTGAACGGCCACGAG
TTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCCACAACACCGTGAAGCTGAAGGTGACCAAGGGCGGC
CCCCTGCCCTTCGCCTGGGACATCCTGTCCCCCCAGTTCCAGTACGGCTCCAAGGTGTACGTGAAGCACCCCGCCGACAT
CCCCGACTACAAGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTG
ACCGTGACCCAGGACTCCTCCCTGCAGGACGGCTGCTTCATCTACAAGGTGAAGTTCATCGGCGTGAACTTCCCCTCCGA
CGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCCGCGACGGCGTGCTGAA
GGGCGAGATCCACAAGGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGTCCATCTACATGGCCAAG
AAGCCCGTGCAGCTGCCCGGCTACTACTACGTGGACTCCAAGCTGGACATCACCTCCCACAACGAGGATTACACCATCGT
GGAGCAGTACGAGCGCACCGAGGGCCGCCACCACCTGTTCCTGTAGCGGCCGCGACTCTAGATCATAATCAGCCATACCA
CATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACTTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTT
GTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTG
CATTCTAGTTGTGGTTTGTCCAAACTCATCATGTATCTTAAGCTTATCGATACGCGTACGGCGCGCCTAGGCCGGCCGAATT
CGAATGGCCATGGGACGTCGACCTGAGGTAATTATAACCCGGGCCCTATATATGGATCCAATTGCAATGATCATCATGACA
GATCTGCGCGCGATCGATATCTGCAGGTCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCT
CCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGACTCTAGCGAGCGCCGGAGATCCGTTTCATAT
ATAAGCGCGGTCTCGCGGCGCGTTGTCAGTGAATTTGAATCAAGCGAAGCGAAAGCAACAAAGCTAAGCAAGCGACTCAA
AGCAAATTTTCAAAGCGACTTTAACAAAGTTGAGTGAATTATTTACTAGTGAATTCAAAGTGGTACCATGTCTTTTCAACAAA
CAAACAACAACGCAACCAACAACATCAACTCCCTTGAGGAGCTTGCTGCTCAAGAACTAATAGCAGCACAATTTGAAGGAAA
TCTTGATGGTTTCTTTTGCACTTTTTATGTGCAGTCCAAACCACAACTATTGGACTTAGAGAGTGAATGTTATTGTATGGATG
ATTTTGATTGTGGGTGTGATAGGATCAAGAGAGAAGAAGAATTACGTAAACTGATTTTCTTAACATCGGACGTTTATGGATAT
AACTTTGAAGAGTGGAAAGGATTAGTTTGGAAATTTGTTCAAAATTATTGCCCAGAACATCGATATGGATCAACTTTTGGTAA
TGGATTATTAATTGTGAGTCCCCGTTTCTTTATGGATCATCTTGACTGGTTTCAGCAATGGAAACTTGTTTCAAGTAATGATG
AATGCAGAGCCTTCAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAA
CCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTT
ACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCA
ATGTATCTTAAGCTTGGCGCGCCTAGGCCGGCCGATCTCGGATCTGACAATGTTCAGTGCAGAGACTCGGCTACGCCTCG
TGGACTTTGAAGTTGACCAACAATGTTTATTCTTACCTCTAATAGTCCTCTGTGGCAAGGTCAAGATTCTGTTAGAAGCCAAT
GAAGAACCTGGTTGTTCAATAACATTTTGTTCGTCTAATATTTCACTACCGCTTGACGTTGGCTGCACTTCATGTACCTCATC
TATAAACGCTTCTTCTGTATCGCTCTGGACGTCATCTTCACTTACGTGATCTGATATTTCACTGTCAGAATCCTCACCAACAA
GCTCGTCATCGCTTTGCAGAAGAGCAGAGAGGATATGCTCATCGTCTAAAGAACTACCCATTTTATTATATATTAGTCACGA
TATCTATAACAAGAAAATATATATATAATAAGTTATCACGTAAGTAGAACATGAAATAACAATATAATTATCGTATGAGTTAAAT
CTTAAAAGTCACGTAAAAGATAATCATGCGTCATTTTGACTCACGCGGTCGTTATAGTTCAAAATCAGTGACACTTACCGCAT
TGACAAGCACGCCTCACGGGAGCTCCAAGCGGCGACTGAGATGTCCTAAATGCACAGCGACGGATTCGCGCTATTTAGAA
AGAGAGAGCAATATTTCAAGAATGCATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAAAAAAGATTTGCGCTTTACTC
GACCTAAACTTTAAACACGTCATAGAATCTTCGTTTGACAAAAACCACATTGTGGCCAAGCTGTGTGACGCGACGCGCGCT
AAAGAATGGCAAACCAAGTCGCGCGAGCGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTAATCATGGTCA
TAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGG
GTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGC
TGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCG
CTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGA
TAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCC
ATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGA
TACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTT
CTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTG
GGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAG
ACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCT
TGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAA
AAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGC

GCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAG
GGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTA
TATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCC
ATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCG
CGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTG
CAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAA
CGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATC
AAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTT
GGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTG
ACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGAT
AATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTAC
CGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGG
GTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCT
TTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAAT
AGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAAT
AGGCGTATCACGAGGCCCTTTCGTC

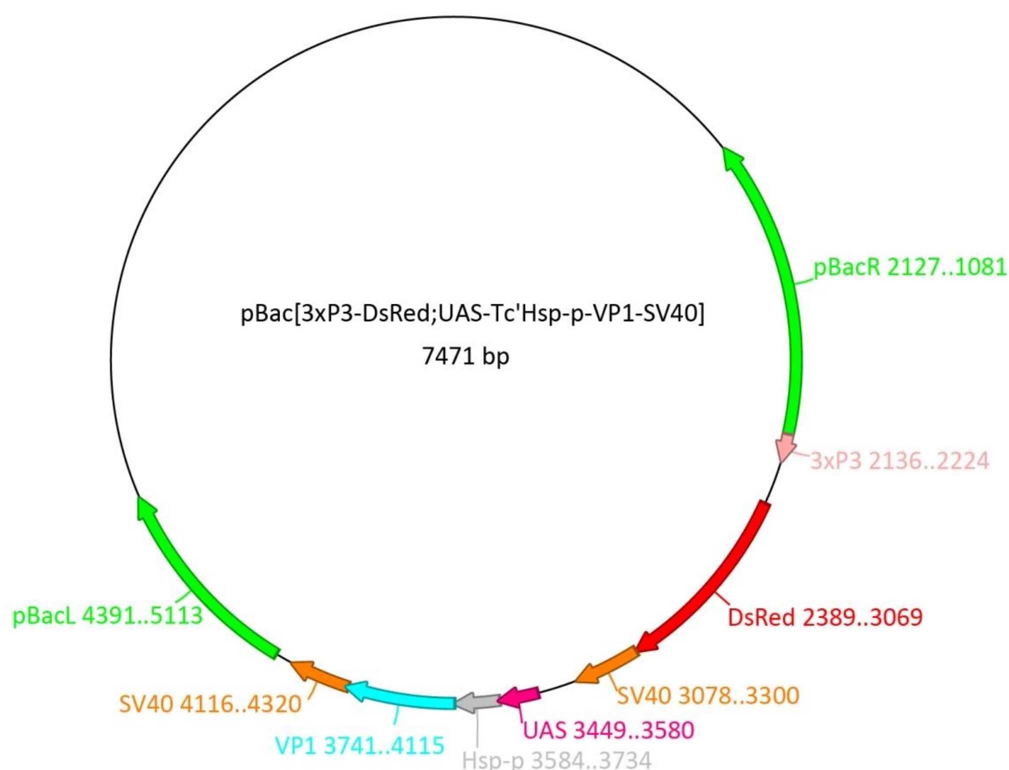


>pBac3xP3-DsRed;UAS-Tc'Hsp-p-B2-SV40

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGAT
GCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCA
GAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCG
CCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAG
GGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCC
AAGCTTTGTTTAAAATATAACAAAATTGTGATCCCACAAAATGAAGTGGGGCAAAATCAAATAATTAACTAGTGTCCGTAAAC
TTGTTGGTCTTCAACTTTTTGAGGAACACGTTGGACGGCAAATCGTGACTATAACACAAGTTGATTTAATAATTTTAGCCAAC
ACGTCGGGCTGCGTGTTTTTTGCGCTCTGTGTACACGTTGATTAACTGGTCGATTAAATAATTTAATTTTTGGTTCTTCTTTAA
ATCTGTGATGAAATTTTTTAAAATAACTTTAAATTCTTCATTGGTAAAAAATGCCACGTTTTGCAACTTGTGAGGGTCTAATAT
GAGGTCAAACTCAGTAGGAGTTTTATCCAAAAAAGAAAACATGATTACGTCTGTACACGAACGCGTATTAACGCAGAGTGCA
AAGTATAAGAGGGTTAAAAAATATATTTTACGCACCATATACGCATCGGGTTGATATCGTTAATATGGATCAATTTGAACAGT

TGATTAACGTGTCTCTGCTCAAGTCTTTGATCAAAACGCAAATCGACGAAAATGTGTCGGACAATATCAAGTCGATGAGCGA
AAAACTAAAAAGGCTAGAATACGACAATCTCACAGACAGCGTTGAGATATACGGTATTCACGACAGCAGGCTGAATAATAAA
AAAATTAGAAACTATTATTTAACCCTAGAAAGATAATCATATTGTGACGTACGTTAAAGATAATCATGCGTAAAATTGACGCAT
GTGTTTTATCGGTCTGTATATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTTATTATATTTACACTTACATACTAATA
ATAAATTCAACAAACAATTTATTTATGTTTATTTATTTATTAAAAAAAAACAAAAACTCAAAATTTCTTCTATAAAGTAACAAAAC
TTTTAAACATTCTCTCTTTTACAAAAATAAACTTATTTTGTACTTTAAAAACAGTCATGTTGTATTATAAAATAAGTAATTAGCTT
AACTTATACATAATAGAAACAAATTATACTTATTAGTCAGTCAGAAACAACTTTGGCACATATCAATATTATGCTCTCGACAAA
TAACTTTTTTGCATTTTTTGCACGATGCATTTGCCTTTCGCCTTATTTTAGAGGGGCAGTAAGTACAGTAAGTACGTTTTTTCA
TTACTGGCTCTTCAGTACTGTCATCTGATGTACCAGGCACTTCATTTGGCAAAATATTAGAGATATTATCGCGCAAATATCTC
TTCAAAGTAGGAGCTTCTAAACGCTTACGCATAAACGATGACGTCAGGCTCATGTAAAGGTTTCTCATAAATTTTTTGCGACT
TTGAACCTTTTCTCCCTTGCTACTGACATTATGGCTGTATATAATAAAAGAATTTATGCAGGCAATGTTTATCATTCCGTACAA
TAATGCCATAGGCCACCTATTCGTCTTCCTACTGCAGGTCATCACAGAACACATTTGGTCTAGCGTGTCCACTCCGCCTTTA
GTTTGATTATAATACATAACCATTTGCGGTTTACCGGTACTTTCGTTGATAGAAGCATCCTCATCACAAGATGATAATAAGTA
TACCATCTTAGCTGGCTTCGGTTTATATGAGACGAGAGTAAGGGGTCCGTCAAAACAAAACATCGATGTTCCCACTGGCCT
GGAGCGACTGTTTTTCAGTACTTCCGGTATCTCGCGTTTGTTTGATCGCACGGTTCCCACAATGCGGGGATTATTCATTAGA
GACTAATTCAATTAGAGCTAATTCAATTAGGATCCAAGCTTATCGATTTCGAACCCTCGACCGCCGGAGTATAAATAGAGGC
GCTTCGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTAAGCAAATAAACAAGC
GCAGCTGAACAAGCTAAACAATCGGGGTACCGCTAGAGTCGACGGTACCGCGGGCCCGGGATCCACCGGTCGCCACCAT
GGTGCGCTCCTCCAAGAACGTCATCAAGGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCACCGTGAACGGCCACGAG
TTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCCACAACACCGTGAAGCTGAAGGTGACCAAGGGCGGC
CCCCTGCCCTTCGCCTGGGACATCCTGTCCCCCCAGTTCCAGTACGGCTCCAAGGTGTACGTGAAGCACCCCGCCGACAT
CCCCGACTACAAGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTG
ACCGTGACCCAGGACTCCTCCCTGCAGGACGGCTGCTTCATCTACAAGGTGAAGTTCATCGGCGTGAACTTCCCCTCCGA
CGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCCGCGACGGCGTGCTGAA
GGGCGAGATCCACAAGGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGTCCATCTACATGGCCAAG
AAGCCCGTGCAGCTGCCCGGCTACTACTACGTGGACTCCAAGCTGGACATCACCTCCCACAACGAGGATTACACCATCGT
GGAGCAGTACGAGCGCACCGAGGGCCGCCACCACCTGTTCCTGTAGCGGCCGCGACTCTAGATCATAATCAGCCATACCA
CATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACTTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTT
GTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTG
CATTCTAGTTGTGGTTTGTCCAAACTCATCATGTATCTTAAGCTTATCGATACGCGTACGGCGCGCCTAGGCCGGCCGAATT
CGAATGGCCATGGGACGTCGACCTGAGGTAATTATAACCCGGGCCCTATATATGGATCCAATTGCAATGATCATCATGACA
GATCTGCGCGCGATCGATATCTGCAGGTCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCT
CCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGACTCTAGCGAGCGCCGGAGATCCGTTTCATAT
ATAAGCGCGGTCTCGCGGCGCGTTGTCAGTGAATTTGAATCAAGCGAAGCGAAAGCAACAAAGCTAAGCAAGCGACTCAA
AGCAAATTTTCAAAGCGACTTTAACAAAGTTGAGTGAATTATTTACTAGTGAATTCAAAGTGGTACCATGCCAAGCAAACTCG
CGCTAATCCAGGAACTTCCCGACCGCATTCAAACGGCGGTGGAAGCAGCCATGGGAATGAGCTACCAAGACGCACCGAAC
AACGTGCGCAGGGACCTCGACAACCTGCACGCTTGCCTAAACAAGGCAAAACTAACGGTAAGTCGGATGGTAACATCACT
GCTGGAGAAACCCAGCGTGGTGGCATACCTAGAGGGAAAGGCCCCCGAGGAGGCAAAACCAACACTCGAAGAACGCCTC
CGAAAGCTGGAGCTCAGCCACAGCCTTCCAACAACCGGAAGTGACCCCCCACCCGCAAAACTGAAGCGGCCGCGACTCTA
GATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATA
AAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAA
ATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTTAAGCTTGGCGCGCCTAGGCCGG
CCGATCTCGGATCTGACAATGTTCAGTGCAGAGACTCGGCTACGCCTCGTGGACTTTGAAGTTGACCAACAATGTTTATTCT
TACCTCTAATAGTCCTCTGTGGCAAGGTCAAGATTCTGTTAGAAGCCAATGAAGAACCTGGTTGTTCAATAACATTTTGTTCG
TCTAATATTTCACTACCGCTTGACGTTGGCTGCACTTCATGTACCTCATCTATAAACGCTTCTTCTGTATCGCTCTGGACGTC
ATCTTCACTTACGTGATCTGATATTTCACTGTCAGAATCCTCACCAACAAGCTCGTCATCGCTTTGCAGAAGAGCAGAGAGG
ATATGCTCATCGTCTAAAGAACTACCCATTTTATTATATATTAGTCACGATATCTATAACAAGAAAATATATATATAATAAGTTA
TCACGTAAGTAGAACATGAAATAACAATATAATTATCGTATGAGTTAAATCTTAAAAGTCACGTAAAAGATAATCATGCGTCAT
TTTGACTCACGCGGTCGTTATAGTTCAAAATCAGTGACACTTACCGCATTGACAAGCACGCCTCACGGGAGCTCCAAGCGG
CGACTGAGATGTCCTAAATGCACAGCGACGGATTCGCGCTATTTAGAAAGAGAGAGCAATATTTCAAGAATGCATGCGTCA
ATTTTACGCAGACTATCTTTCTAGGGTTAAAAAAGATTTGCGCTTTACTCGACCTAAACTTTAAACACGTCATAGAATCTTCG

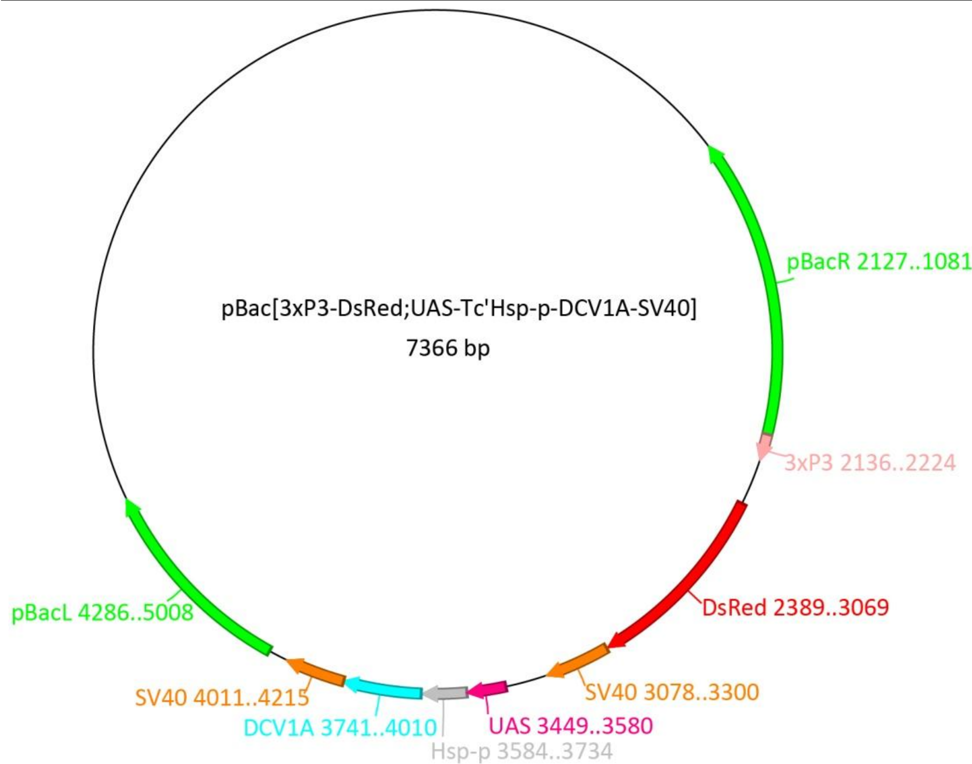
TTTGACAAAAACCACATTGTGGCCAAGCTGTGTGACGCGACGCGCGCTAAAGAATGGCAAACCAAGTCGCGCGAGCGTCG
ACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCA
CAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTG
CGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGA
GGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGT
ATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCA
GCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAA
ATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTG
CGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGC
TCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGA
CCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTG
GTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGA
AGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAA
ACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTG
ATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCT
TCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAAT
GCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAAC
TACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTAT
CAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATT
GTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGT
CACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCA
AAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAG
CACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAA
TAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTG
CTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTC
GTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAA
AAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTAT
TGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGC
CACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTC



>pBac3xP3-DsRed;UAS-Tc'Hsp-p-VP1-SV40

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGAT
GCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCA
GAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCG
CCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAG
GGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCC
AAGCTTTGTTTAAAATATAACAAAATTGTGATCCCACAAAATGAAGTGGGGCAAAATCAAATAATTAACTAGTGTCCGTAAAC
TTGTTGGTCTTCAACTTTTTGAGGAACACGTTGGACGGCAAATCGTGACTATAACACAAGTTGATTTAATAATTTTAGCCAAC
ACGTCGGGCTGCGTGTTTTTTGCGCTCTGTGTACACGTTGATTAACTGGTCGATTAAATAATTTAATTTTTGGTTCTTCTTTAA
ATCTGTGATGAAATTTTTTAAAATAACTTTAAATTCTTCATTGGTAAAAAATGCCACGTTTTGCAACTTGTGAGGGTCTAATAT
GAGGTCAAACTCAGTAGGAGTTTTATCCAAAAAAGAAAACATGATTACGTCTGTACACGAACGCGTATTAACGCAGAGTGCA
AAGTATAAGAGGGTTAAAAAATATATTTTACGCACCATATACGCATCGGGTTGATATCGTTAATATGGATCAATTTGAACAGT
TGATTAACGTGTCTCTGCTCAAGTCTTTGATCAAAACGCAAATCGACGAAAATGTGTCGGACAATATCAAGTCGATGAGCGA
AAAACTAAAAAGGCTAGAATACGACAATCTCACAGACAGCGTTGAGATATACGGTATTCACGACAGCAGGCTGAATAATAAA
AAAATTAGAAACTATTATTTAACCCTAGAAAGATAATCATATTGTGACGTACGTTAAAGATAATCATGCGTAAAATTGACGCAT
GTGTTTTATCGGTCTGTATATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTTATTATATTTACACTTACATACTAATA
ATAAATTCAACAAACAATTTATTTATGTTTATTTATTTATTAAAAAAAAACAAAAACTCAAAATTTCTTCTATAAAGTAACAAAAC
TTTTAAACATTCTCTCTTTTACAAAAATAAACTTATTTTGTACTTTAAAAACAGTCATGTTGTATTATAAAATAAGTAATTAGCTT
AACTTATACATAATAGAAACAAATTATACTTATTAGTCAGTCAGAAACAACTTTGGCACATATCAATATTATGCTCTCGACAAA
TAACTTTTTTGCATTTTTTGCACGATGCATTTGCCTTTCGCCTTATTTTAGAGGGGCAGTAAGTACAGTAAGTACGTTTTTTCA
TTACTGGCTCTTCAGTACTGTCATCTGATGTACCAGGCACTTCATTTGGCAAAATATTAGAGATATTATCGCGCAAATATCTC
TTCAAAGTAGGAGCTTCTAAACGCTTACGCATAAACGATGACGTCAGGCTCATGTAAAGGTTTCTCATAAATTTTTTGCGACT
TTGAACCTTTTCTCCCTTGCTACTGACATTATGGCTGTATATAATAAAAGAATTTATGCAGGCAATGTTTATCATTCCGTACAA
TAATGCCATAGGCCACCTATTCGTCTTCCTACTGCAGGTCATCACAGAACACATTTGGTCTAGCGTGTCCACTCCGCCTTTA
GTTTGATTATAATACATAACCATTTGCGGTTTACCGGTACTTTCGTTGATAGAAGCATCCTCATCACAAGATGATAATAAGTA
TACCATCTTAGCTGGCTTCGGTTTATATGAGACGAGAGTAAGGGGTCCGTCAAAACAAAACATCGATGTTCCCACTGGCCT
GGAGCGACTGTTTTTCAGTACTTCCGGTATCTCGCGTTTGTTTGATCGCACGGTTCCCACAATGCGGGGATTATTCATTAGA
GACTAATTCAATTAGAGCTAATTCAATTAGGATCCAAGCTTATCGATTTCGAACCCTCGACCGCCGGAGTATAAATAGAGGC
GCTTCGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTAAGCAAATAAACAAGC
GCAGCTGAACAAGCTAAACAATCGGGGTACCGCTAGAGTCGACGGTACCGCGGGCCCGGGATCCACCGGTCGCCACCAT
GGTGCGCTCCTCCAAGAACGTCATCAAGGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCACCGTGAACGGCCACGAG
TTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCCACAACACCGTGAAGCTGAAGGTGACCAAGGGCGGC
CCCCTGCCCTTCGCCTGGGACATCCTGTCCCCCCAGTTCCAGTACGGCTCCAAGGTGTACGTGAAGCACCCCGCCGACAT
CCCCGACTACAAGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTG
ACCGTGACCCAGGACTCCTCCCTGCAGGACGGCTGCTTCATCTACAAGGTGAAGTTCATCGGCGTGAACTTCCCCTCCGA
CGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCCGCGACGGCGTGCTGAA
GGGCGAGATCCACAAGGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGTCCATCTACATGGCCAAG
AAGCCCGTGCAGCTGCCCGGCTACTACTACGTGGACTCCAAGCTGGACATCACCTCCCACAACGAGGATTACACCATCGT
GGAGCAGTACGAGCGCACCGAGGGCCGCCACCACCTGTTCCTGTAGCGGCCGCGACTCTAGATCATAATCAGCCATACCA
CATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACTTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTT
GTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTG
CATTCTAGTTGTGGTTTGTCCAAACTCATCATGTATCTTAAGCTTATCGATACGCGTACGGCGCGCCTAGGCCGGCCGAATT
CGAATGGCCATGGGACGTCGACCTGAGGTAATTATAACCCGGGCCCTATATATGGATCCAATTGCAATGATCATCATGACA
GATCTGCGCGCGATCGATATCTGCAGGTCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCT
CCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGACTCTAGCGAGCGCCGGAGATCCGTTTCATAT
ATAAGCGCGGTCTCGCGGCGCGTTGTCAGTGAATTTGAATCAAGCGAAGCGAAAGCAACAAAGCTAAGCAAGCGACTCAA
AGCAAATTTTCAAAGCGACTTTAACAAAGTTGAGTGAATTATTTACTAGTGAATTCAAAGTGGTACCCCACCAGCAGAGGAG
AAAAGGAGTATCCATTTTTATGGTTACAAACCAAAAGGAATCCCTAACAAAGTTTGGTGGAACTGGGTCACCACTGGCACAG
CTATGGACGCTTATGAAAAAGCTGACCGTTATCTGTATCATCAATTTAAACGAGAAATGATGATATACAGAAATAAATGGGTC
AAATTTAGTAAGGAGTTCAATCCATACCTATCAAAACCGAAAATGGTGTGGGAAGAGAATACATGGGAATATGAATATAAAA
CGGACGTTCCCTATAATTTTATTCTCAAATGGCGCCAGTTAGTACAGACTTACAAACCTAACACACCAATCCAGGCTGATTG

GTATAAAATCTCGCAGAAACAACAATGTTAAAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTT
TTACTTGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATT
GCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGT
TTGTCCAAACTCATCAATGTATCTTAAGCTTGGCGCGCCTAGGCCGGCCGATCTCGGATCTGACAATGTTCAGTGCAGAGA
CTCGGCTACGCCTCGTGGACTTTGAAGTTGACCAACAATGTTTATTCTTACCTCTAATAGTCCTCTGTGGCAAGGTCAAGAT
TCTGTTAGAAGCCAATGAAGAACCTGGTTGTTCAATAACATTTTGTTCGTCTAATATTTCACTACCGCTTGACGTTGGCTGCA
CTTCATGTACCTCATCTATAAACGCTTCTTCTGTATCGCTCTGGACGTCATCTTCACTTACGTGATCTGATATTTCACTGTCA
GAATCCTCACCAACAAGCTCGTCATCGCTTTGCAGAAGAGCAGAGAGGATATGCTCATCGTCTAAAGAACTACCCATTTTAT
TATATATTAGTCACGATATCTATAACAAGAAAATATATATATAATAAGTTATCACGTAAGTAGAACATGAAATAACAATATAATT
ATCGTATGAGTTAAATCTTAAAAGTCACGTAAAAGATAATCATGCGTCATTTTGACTCACGCGGTCGTTATAGTTCAAAATCA
GTGACACTTACCGCATTGACAAGCACGCCTCACGGGAGCTCCAAGCGGCGACTGAGATGTCCTAAATGCACAGCGACGGA
TTCGCGCTATTTAGAAAGAGAGAGCAATATTTCAAGAATGCATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAAAAAA
GATTTGCGCTTTACTCGACCTAAACTTTAAACACGTCATAGAATCTTCGTTTGACAAAAACCACATTGTGGCCAAGCTGTGT
GACGCGACGCGCGCTAAAGAATGGCAAACCAAGTCGCGCGAGCGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAA
TTCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATA
AAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGA
AACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTC
CTCGCTCACTGACTCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTAT
CCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGC
GTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACC
CGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACC
GGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAG
GTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCT
TGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAG
GCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGA
AGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTT
GCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGA
ACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGT
TTTAAATCAATCTAAAGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGAT
CTGTCTATTTCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCC
CAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCG
AGCGCAGAAGTGGTCCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGC
CAGTTAATAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAG
CTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGAT
CGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCC
GTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGC
CCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGA
AAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTA
CTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGT
TGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGT
ATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCA
TGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTC

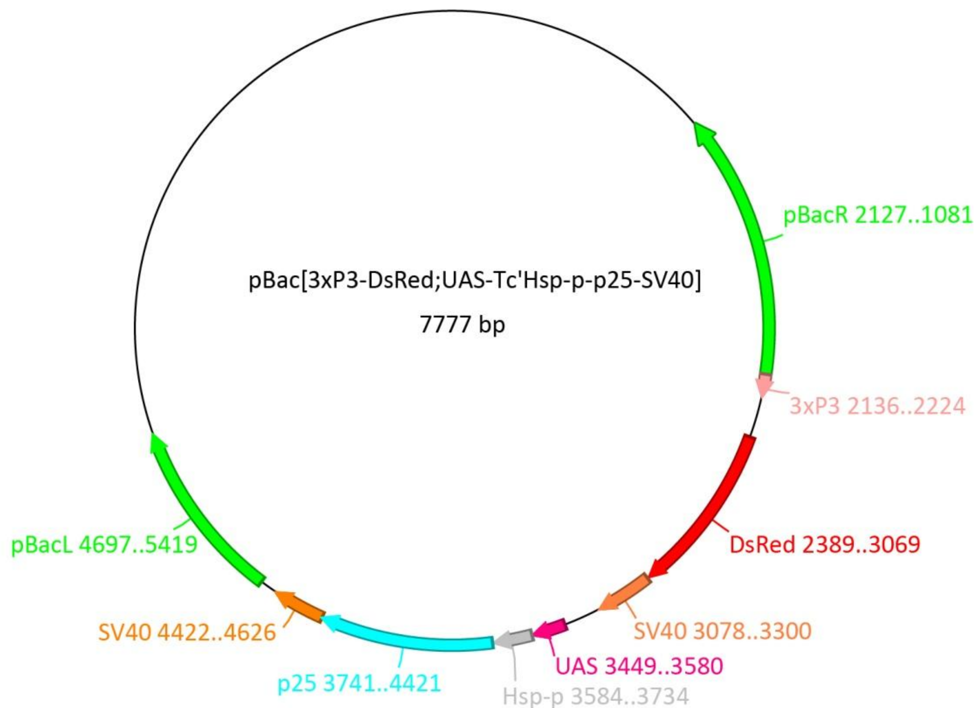


>pBac3xP3-DsRed;UAS-Tc'Hsp-p-DCV1A-SV40

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGAT
GCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCA
GAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCG
CCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAG
GGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCC
AAGCTTTGTTTAAAATATAACAAAATTGTGATCCCACAAAATGAAGTGGGGCAAAATCAAATAATTAACTAGTGTCCGTAAAC
TTGTTGGTCTTCAACTTTTTGAGGAACACGTTGGACGGCAAATCGTGACTATAACACAAGTTGATTTAATAATTTTAGCCAAC
ACGTCGGGCTGCGTGTTTTTTGCGCTCTGTGTACACGTTGATTAACTGGTCGATTAAATAATTTAATTTTTGGTTCTTCTTTAA
ATCTGTGATGAAATTTTTTAAAATAACTTTAAATTCTTCATTGGTAAAAAATGCCACGTTTTGCAACTTGTGAGGGTCTAATAT
GAGGTCAAACTCAGTAGGAGTTTTATCCAAAAAAGAAAACATGATTACGTCTGTACACGAACGCGTATTAACGCAGAGTGCA
AAGTATAAGAGGGTTAAAAAATATATTTTACGCACCATATACGCATCGGGTTGATATCGTTAATATGGATCAATTTGAACAGT
TGATTAACGTGTCTCTGCTCAAGTCTTTGATCAAAACGCAAATCGACGAAAATGTGTCGGACAATATCAAGTCGATGAGCGA
AAAACTAAAAAGGCTAGAATACGACAATCTCACAGACAGCGTTGAGATATACGGTATTCACGACAGCAGGCTGAATAATAAA
AAAATTAGAAACTATTATTTAACCCTAGAAAGATAATCATATTGTGACGTACGTTAAAGATAATCATGCGTAAAATTGACGCAT
GTGTTTTATCGGTCTGTATATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTTATTATATTTACACTTACATACTAATA
ATAAATTCAACAAACAATTTATTTATGTTTATTTATTTATTAAAAAAAAACAAAAACTCAAAATTTCTTCTATAAAGTAACAAAAC
TTTTAAACATTCTCTCTTTTACAAAAATAAACTTATTTTGTACTTTAAAAACAGTCATGTTGTATTATAAAATAAGTAATTAGCTT
AACTTATACATAATAGAAACAAATTATACTTATTAGTCAGTCAGAAACAACTTTGGCACATATCAATATTATGCTCTCGACAAA
TAACTTTTTTGCATTTTTTGCACGATGCATTTGCCTTTCGCCTTATTTTAGAGGGGCAGTAAGTACAGTAAGTACGTTTTTTCA
TTACTGGCTCTTCAGTACTGTCATCTGATGTACCAGGCACTTCATTTGGCAAAATATTAGAGATATTATCGCGCAAATATCTC
TTCAAAGTAGGAGCTTCTAAACGCTTACGCATAAACGATGACGTCAGGCTCATGTAAAGGTTTCTCATAAATTTTTTGCGACT
TTGAACCTTTTCTCCCTTGCTACTGACATTATGGCTGTATATAATAAAAGAATTTATGCAGGCAATGTTTATCATTCCGTACAA
TAATGCCATAGGCCACCTATTCGTCTTCCTACTGCAGGTCATCACAGAACACATTTGGTCTAGCGTGTCCACTCCGCCTTTA
GTTTGATTATAATACATAACCATTTGCGGTTTACCGGTACTTTCGTTGATAGAAGCATCCTCATCACAAGATGATAATAAGTA
TACCATCTTAGCTGGCTTCGGTTTATATGAGACGAGAGTAAGGGGTCCGTCAAAACAAAACATCGATGTTCCCACTGGCCT
GGAGCGACTGTTTTTCAGTACTTCCGGTATCTCGCGTTTGTTTGATCGCACGGTTCCCACAATGCGGGGATTATTCATTAGA
GACTAATTCAATTAGAGCTAATTCAATTAGGATCCAAGCTTATCGATTTCGAACCCTCGACCGCCGGAGTATAAATAGAGGC
GCTTCGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTAAGCAAATAAACAAGC

GCAGCTGAACAAGCTAAACAATCGGGGTACCGCTAGAGTCGACGGTACCGCGGGCCCGGGATCCACCGGTCGCCACCAT
GGTGCGCTCCTCCAAGAACGTCATCAAGGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCACCGTGAACGGCCACGAG
TTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCCACAACACCGTGAAGCTGAAGGTGACCAAGGGCGGC
CCCCTGCCCTTCGCCTGGGACATCCTGTCCCCCCAGTTCCAGTACGGCTCCAAGGTGTACGTGAAGCACCCCGCCGACAT
CCCCGACTACAAGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTG
ACCGTGACCCAGGACTCCTCCCTGCAGGACGGCTGCTTCATCTACAAGGTGAAGTTCATCGGCGTGAACTTCCCCTCCGA
CGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCCGCGACGGCGTGCTGAA
GGGCGAGATCCACAAGGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGTCCATCTACATGGCCAAG
AAGCCCGTGCAGCTGCCCGGCTACTACTACGTGGACTCCAAGCTGGACATCACCTCCCACAACGAGGATTACACCATCGT
GGAGCAGTACGAGCGCACCGAGGGCCGCCACCACCTGTTCCTGTAGCGGCCGCGACTCTAGATCATAATCAGCCATACCA
CATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACTTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTT
GTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTG
CATTCTAGTTGTGGTTTGTCCAAACTCATCATGTATCTTAAGCTTATCGATACGCGTACGGCGCGCCTAGGCCGGCCGAATT
CGAATGGCCATGGGACGTCGACCTGAGGTAATTATAACCCGGGCCCTATATATGGATCCAATTGCAATGATCATCATGACA
GATCTGCGCGCGATCGATATCTGCAGGTCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCT
CCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGACTCTAGCGAGCGCCGGAGATCCGTTTCATAT
ATAAGCGCGGTCTCGCGGCGCGTTGTCAGTGAATTTGAATCAAGCGAAGCGAAAGCAACAAAGCTAAGCAAGCGACTCAA
AGCAAATTTTCAAAGCGACTTTAACAAAGTTGAGTGAATTATTTACTAGTGAATTCAAAGTGGTACCATGGAATCTGATAAAA
GTATGGCCTGTTTAAATAGAATTTTGATGAACAAGATGATGTTTGTGGAAGATAAGATCTCCACCCTTAAGATGGTTGCTGAT
TATTATCAAAAAGAAGTAAAGTATGATTTTGATGCAGTTGAATCTCCCCGTGAGGCACCCGTATTTAAATGTACTTGTAGATT
TCTTGGTTATACCATTATGACTCAAGGCATCGGTAAGAAGAATCCGAAACAGGAAGCTGCACGTCAGATGTTGCTCTTGTTA
TCAGGAAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCAC
ACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAA
GCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCATCAATGTATCTT
AAGCTTGGCGCGCCTAGGCCGGCCGATCTCGGATCTGACAATGTTCAGTGCAGAGACTCGGCTACGCCTCGTGGACTTTG
AAGTTGACCAACAATGTTTATTCTTACCTCTAATAGTCCTCTGTGGCAAGGTCAAGATTCTGTTAGAAGCCAATGAAGAACCT
GGTTGTTCAATAACATTTTGTTCGTCTAATATTTCACTACCGCTTGACGTTGGCTGCACTTCATGTACCTCATCTATAAACGC
TTCTTCTGTATCGCTCTGGACGTCATCTTCACTTACGTGATCTGATATTTCACTGTCAGAATCCTCACCAACAAGCTCGTCAT
CGCTTTGCAGAAGAGCAGAGAGGATATGCTCATCGTCTAAAGAACTACCCATTTTATTATATATTAGTCACGATATCTATAAC
AAGAAAATATATATATAATAAGTTATCACGTAAGTAGAACATGAAATAACAATATAATTATCGTATGAGTTAAATCTTAAAAGT
CACGTAAAAGATAATCATGCGTCATTTTGACTCACGCGGTCGTTATAGTTCAAAATCAGTGACACTTACCGCATTGACAAGC
ACGCCTCACGGGAGCTCCAAGCGGCGACTGAGATGTCCTAAATGCACAGCGACGGATTCGCGCTATTTAGAAAGAGAGAG
CAATATTTCAAGAATGCATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAAAAAAGATTTGCGCTTTACTCGACCTAAA
CTTTAAACACGTCATAGAATCTTCGTTTGACAAAAACCACATTGTGGCCAAGCTGTGTGACGCGACGCGCGCTAAAGAATG
GCAAACCAAGTCGCGCGAGCGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTAATCATGGTCATAGCTGTT
TCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTA
ATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTA
ATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCGCT
CGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCA
GGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCT
CCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAGG
CGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGCCTTTCTCCCTT
CGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGT
GTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGA
CTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGT
GGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAG
TTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAA
AAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTT
GGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGA
GTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATAGTTG
CCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGAC

CCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTT
ATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGCGCAACGTTGTT
GCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGA
GTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCA
GTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTG
AGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCG
CGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTT
GAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCA
AAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAAT
ATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTT
CCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTA
TCACGAGGCCCTTTCGTC

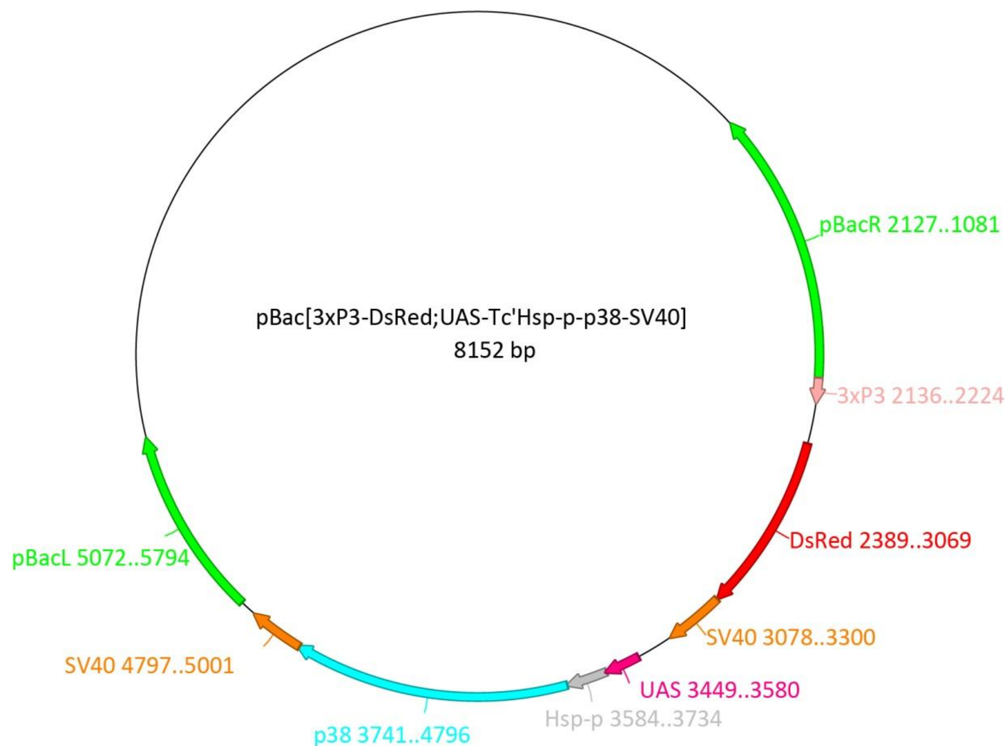


>pBac3xP3-DsRed;UAS-Tc'Hsp-p-p25-SV40

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCACAGCTTGTCTGTAAGCGGAT
GCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTGTTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCA
GAGCAGATTGTACTGAGAGTGCACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCG
CCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAAG
GGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCC
AAGCTTTGTTTAAAATATAACAAAATTGTGATCCCACAAAATGAAGTGGGGCAAAATCAAATAATTAACTAGTGTCCGTAAAC
TTGTTGGTCTTCAACTTTTTGAGGAACACGTTGGACGGCAAATCGTGACTATAACACAAGTTGATTTAATAATTTTAGCCAAC
ACGTCGGGCTGCGTGTTTTTTGCGCTCTGTGTACACGTTGATTAACTGGTCGATTAAATAATTTAATTTTTGGTTCTTCTTTAA
ATCTGTGATGAAATTTTTTAAAATAACTTTAAATTCTTCATTGGTAAAAAATGCCACGTTTTGCAACTTGTGAGGGTCTAATAT
GAGGTCAAACTCAGTAGGAGTTTTATCCAAAAAAGAAAACATGATTACGTCTGTACACGAACGCGTATTAACGCAGAGTGCA
AAGTATAAGAGGGTTAAAAAATATATTTTACGCACCATATACGCATCGGGTTGATATCGTTAATATGGATCAATTTGAACAGT
TGATTAACGTGTCTCTGCTCAAGTCTTTGATCAAAACGCAAATCGACGAAAATGTGTCGGACAATATCAAGTCGATGAGCGA
AAAACTAAAAAGGCTAGAATACGACAATCTCACAGACAGCGTTGAGATATACGGTATTCACGACAGCAGGCTGAATAATAAA
AAAATTAGAAACTATTATTTAACCCTAGAAAGATAATCATATTGTGACGTACGTTAAAGATAATCATGCGTAAAATTGACGCAT
GTGTTTTATCGGTCTGTATATCGAGGTTTATTTATTAATTTGAATAGATATTAAGTTTTATTATATTTACACTTACATACTAATA
ATAAATTCAACAAACAATTTATTTATGTTTATTTATTTATTAAAAAAAAACAAAAACTCAAAATTTCTTCTATAAAGTAACAAAAC

TTTTAAACATTCTCTCTTTTACAAAAATAAACTTATTTTGTACTTTAAAAACAGTCATGTTGTATTATAAAATAAGTAATTAGCTT
AACTTATACATAATAGAAACAAATTATACTTATTAGTCAGTCAGAAACAACTTTGGCACATATCAATATTATGCTCTCGACAAA
TAACTTTTTTGCATTTTTTGCACGATGCATTTGCCTTTCGCCTTATTTTAGAGGGGCAGTAAGTACAGTAAGTACGTTTTTTCA
TTACTGGCTCTTCAGTACTGTCATCTGATGTACCAGGCACTTCATTTGGCAAAATATTAGAGATATTATCGCGCAAATATCTC
TTCAAAGTAGGAGCTTCTAAACGCTTACGCATAAACGATGACGTCAGGCTCATGTAAAGGTTTCTCATAAATTTTTTGCGACT
TTGAACCTTTTCTCCCTTGCTACTGACATTATGGCTGTATATAATAAAAGAATTTATGCAGGCAATGTTTATCATTCCGTACAA
TAATGCCATAGGCCACCTATTCGTCTTCCTACTGCAGGTCATCACAGAACACATTTGGTCTAGCGTGTCCACTCCGCCTTTA
GTTTGATTATAATACATAACCATTTGCGGTTTACCGGTACTTTCGTTGATAGAAGCATCCTCATCACAAGATGATAATAAGTA
TACCATCTTAGCTGGCTTCGGTTTATATGAGACGAGAGTAAGGGGTCCGTCAAAACAAAACATCGATGTTCCCACTGGCCT
GGAGCGACTGTTTTTCAGTACTTCCGGTATCTCGCGTTTGTTTGATCGCACGGTTCCCACAATGCGGGGATTATTCATTAGA
GACTAATTCAATTAGAGCTAATTCAATTAGGATCCAAGCTTATCGATTTCGAACCCTCGACCGCCGGAGTATAAATAGAGGC
GCTTCGTCTACGGAGCGACAATTCAATTCAAACAAGCAAAGTGAACACGTCGCTAAGCGAAAGCTAAGCAAATAAACAAGC
GCAGCTGAACAAGCTAAACAATCGGGGTACCGCTAGAGTCGACGGTACCGCGGGCCCGGGATCCACCGGTCGCCACCAT
GGTGCGCTCCTCCAAGAACGTCATCAAGGAGTTCATGCGCTTCAAGGTGCGCATGGAGGGCACCGTGAACGGCCACGAG
TTCGAGATCGAGGGCGAGGGCGAGGGCCGCCCCTACGAGGGCCACAACACCGTGAAGCTGAAGGTGACCAAGGGCGGC
CCCCTGCCCTTCGCCTGGGACATCCTGTCCCCCCAGTTCCAGTACGGCTCCAAGGTGTACGTGAAGCACCCCGCCGACAT
CCCCGACTACAAGAAGCTGTCCTTCCCCGAGGGCTTCAAGTGGGAGCGCGTGATGAACTTCGAGGACGGCGGCGTGGTG
ACCGTGACCCAGGACTCCTCCCTGCAGGACGGCTGCTTCATCTACAAGGTGAAGTTCATCGGCGTGAACTTCCCCTCCGA
CGGCCCCGTAATGCAGAAGAAGACCATGGGCTGGGAGGCCTCCACCGAGCGCCTGTACCCCCGCGACGGCGTGCTGAA
GGGCGAGATCCACAAGGCCCTGAAGCTGAAGGACGGCGGCCACTACCTGGTGGAGTTCAAGTCCATCTACATGGCCAAG
AAGCCCGTGCAGCTGCCCGGCTACTACTACGTGGACTCCAAGCTGGACATCACCTCCCACAACGAGGATTACACCATCGT
GGAGCAGTACGAGCGCACCGAGGGCCGCCACCACCTGTTCCTGTAGCGGCCGCGACTCTAGATCATAATCAGCCATACCA
CATTTGTAGAGGTTTTACTTGCTTTAAAAAACCTCCCACACTTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTT
GTTAACTTGTTTATTGCAGCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTG
CATTCTAGTTGTGGTTTGTCCAAACTCATCATGTATCTTAAGCTTATCGATACGCGTACGGCGCGCCTAGGCCGGCCGAATT
CGAATGGCCATGGGACGTCGACCTGAGGTAATTATAACCCGGGCCCTATATATGGATCCAATTGCAATGATCATCATGACA
GATCTGCGCGCGATCGATATCTGCAGGTCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCT
CCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGACTCTAGCGAGCGCCGGAGATCCGTTTCATAT
ATAAGCGCGGTCTCGCGGCGCGTTGTCAGTGAATTTGAATCAAGCGAAGCGAAAGCAACAAAGCTAAGCAAGCGACTCAA
AGCAAATTTTCAAAGCGACTTTAACAAAGTTGAGTGAATTATTTACTAGTGAATTCAAAGTGGTACCATGGATATTCTCATCA
TTAGTTTGAAAAGTTTAGGTTATTCTAGGACTTCCAAATCTTTAGATTCAGGACCTTTGGTAGTACATGCAGTAGCCGGAGC
CGGTAAGTCCACAGCCCTAAGGAAGTTGATCCTCAGACACCCAACATTCACCGTGCATACACTCGGTGTCCCTGACAAGGT
GAGTATCAGAACTAGAGGCATACAGAAGCCAGGACCTATTCCTGAGGGCAACTTCGCAATCCTCGATGAGTATACTTTGGA
CAACACCACAAGGAACTCATACCAGGCACTTTTTGCTGACCCTTATCAGGCACCGGAGTTTAGCCTAGAGCCCCACTTCTA
CTTGGAAACATCATTTCGAGTTCCGAGGAAAGTGGCAGATTTGATAGCTGGCTGTGGCTTCGATTTCGAGACCAACTCACC
GGAAGAAGGGCACTTAGAGATCACTGGCATATTCAAAGGGCCCCTACTCGGAAAGGTGATAGCCATTGATGAGGAGTCTG
AGACAACACTGTCCAGGCATGGTGTTGAGTTTGTTAAGCCCTGCCAAGTGACGGGACTTGAGTTCAAAGTAGTCACTATTG
TGTCTGCCGCACCAATAGAGGAAATTGGCCAGTCCACAGCTTTCTACAACGCTATCACCAGGTCAAAGGGATTGACATATG
TCCGCGCAGGGACATAGAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTACTTGCTTTAAA
AAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCAGCTTATAATG
GTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTGTCCAAACTCA
TCAATGTATCTTAAGCTTGGCGCGCCTAGGCCGGCCGATCTCGGATCTGACAATGTTCAGTGCAGAGACTCGGCTACGCCT
CGTGGACTTTGAAGTTGACCAACAATGTTTATTCTTACCTCTAATAGTCCTCTGTGGCAAGGTCAAGATTCTGTTAGAAGCC
AATGAAGAACCTGGTTGTTCAATAACATTTTGTTCGTCTAATATTTCACTACCGCTTGACGTTGGCTGCACTTCATGTACCTC
ATCTATAAACGCTTCTTCTGTATCGCTCTGGACGTCATCTTCACTTACGTGATCTGATATTTCACTGTCAGAATCCTCACCAA
CAAGCTCGTCATCGCTTTGCAGAAGAGCAGAGAGGATATGCTCATCGTCTAAAGAACTACCCATTTTATTATATATTAGTCA
CGATATCTATAACAAGAAAATATATATATAATAAGTTATCACGTAAGTAGAACATGAAATAACAATATAATTATCGTATGAGTT
AAATCTTAAAAGTCACGTAAAAGATAATCATGCGTCATTTTGACTCACGCGGTCGTTATAGTTCAAAATCAGTGACACTTACC
GCATTGACAAGCACGCCTCACGGGAGCTCCAAGCGGCGACTGAGATGTCCTAAATGCACAGCGACGGATTCGCGCTATTT
AGAAAGAGAGAGCAATATTTCAAGAATGCATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAAAAAAGATTTGCGCTTT
ACTCGACCTAAACTTTAAACACGTCATAGAATCTTCGTTTGACAAAAACCACATTGTGGCCAAGCTGTGTGACGCGACGCGC

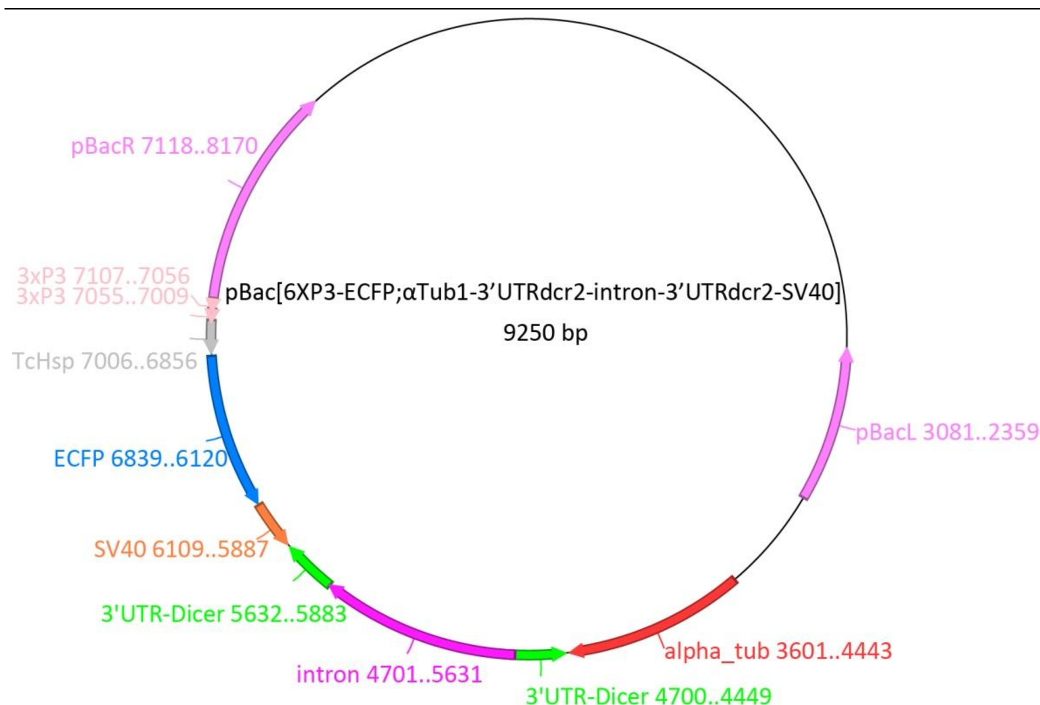
GCTAAAGAATGGCAAACCAAGTCGCGCGAGCGTCGACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCGTAATCATGG
TCATAGCTGTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCT
GGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCC
AGCTGCATTAATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGAC
TCGCTGCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGG
GGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTT
TCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAA
AGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCCGC
CTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAA
GCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGG
TAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAG
TTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTC
GGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATT
ACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGT
TAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAA
AGTATATATGAGTAAACTTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTC
ATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTTACCATCTGGCCCCAGTGCTGCAATGAT
ACCGCGAGACCCACGCTCACCGGCTCCAGATTTATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGT
CCTGCAACTTTATCCGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAATAGTTTGC
GCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAAC
GATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTA
AGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTC
TGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAATACG
GGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGAT
CTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTT
TCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCATACTC
TTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAA
CAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATA
AAAATAGGCGTATCACGAGGCCCTTTCGTC



>pBac3xP3-DsRed;UAS-Tc'Hsp-p-p38-SV40

GACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTT
TCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCT
GATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCA
TTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGT
TACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTA
AAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGA
ATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCA
TAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACA
ACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACC
ACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAA
TAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAAT
CTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATC
TACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGG
TAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATC
CTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAG
GATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTT
GCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGT
GTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGC
TGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCT
GAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCATTGA
GAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACG
AGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTG
TGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCC
TTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTC
GCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCC
CCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATT
AATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCG
GATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACG
CTCGCGCGACTTGGTTTGCCATTCTTTAGCGCGCGTCGCGTCACACAGCTTGGCCACAATGTGGTTTTTGTCAAACGAAGA
TTCTATGACGTGTTTAAAGTTTAGGTCGAGTAAAGCGCAAATCTTTTTTAACCCTAGAAAGATAGTCTGCGTAAAATTGACGC
ATGCATTCTTGAAATATTGCTCTCTCTTTCTAAATAGCGCGAATCCGTCGCTGTGCATTTAGGACATCTCAGTCGCCGCTTG
GAGCTCCCGTGAGGCGTGCTTGTCAATGCGGTAAGTGTCACTGATTTTGAACTATAACGACCGCGTGAGTCAAAATGACGC
ATGATTATCTTTTACGTGACTTTTAAGATTTAACTCATACGATAATTATATTGTTATTTCATGTTCTACTTACGTGATAACTTAT
TATATATATATTTTCTTGTTATAGATATCGTGACTAATATATAATAAAATGGGTAGTTCTTTAGACGATGAGCATATCCTCTCT
GCTCTTCTGCAAAGCGATGACGAGCTTGTTGGTGAGGATTCTGACAGTGAAATATCAGATCACGTAAGTGAAGATGACGTC
CAGAGCGATACAGAAGAAGCGTTTATAGATGAGGTACATGAAGTGCAGCCAACGTCAAGCGGTAGTGAAATATTAGACGAA
CAAAATGTTATTGAACAACCAGGTTCTTCATTGGCTTCTAACAGAATCTTGACCTTGCCACAGAGGACTATTAGAGGTAAGA
ATAAACATTGTTGGTCAACTTCAAAGTCCACGAGGCGTAGCCGAGTCTCTGCACTGAACATTGTCAGATCCGAGATCGGCC
GGCCTAGGCGCGCCTAGGCCGGCCGAATTCGAATGGCCATGGGACGTCGACCTGAGGTAATTATAACCCGGGCCCTATAT
ATGGATCCAATTGCAATGATCATCATGACAGATCTGCGCGCGATCGATATCTGCAGGTCGGAGTACTGTCCTCCGAGCGGA
GTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGCGGAGACT
CTAGCGAGCGCCGGAGATCCGTTTCATATATAAGCGCGGTCTCGCGGCGCGTTGTCAGTGAATTTGAATCAAGCGAAGCG
AAAGCAACAAAGCTAAGCAAGCGACTCAAAGCAAATTTTCAAAGCGACTTTAACAAAGTTGAGTGAATTATTTACTAGTGAAT
TCAAAGTGGTACCATGGAAAATGATCCTAGAGTCCGGAAGTTCGCATCTGATGGCGCCCAATGGGCGATAAAGTGGCAGA
AGAAGGGCTGGTCAACCCTAACCAGCAGACAGAAACAGACCGCCCGCGCAGCGATGGGGATCAAGCTCTCTCCTGTGGC
GCAACCTGTGCAGAAAGTGACTCGGCTGAGTGCTCCGGTGGCCCTTGCCTACCGCGAGGTTTCCACCCAGCCTCGGGTCT
CTACTGCCAGGGACGGCATAACCAGAAGCGGTTCTGAACTGATCACAACCTTGAAGAAGAACACTGACACTGAACCTAAGT
ACACCACAGCTGTGCTTAACCCAAGCGAACCCGGAACATTCAACCAGCTCATTAAGGAGGCGGCCCAGTATGAAAAATACC
GATTCACGTCACTCAGATTTAGGTACTCCCCCATGAGCCCTTCAACCACCGGAGGCAAGGTGGCTCTGGCATTCGACCGA
GATGCAGCCAAACCTCCGCCCAACGACCTCGCTTCCCTCTACAACATAGAGGGTTGTGTATCTAGCGTGCCTTGGACAGG

GTTTATTTTGACCGTCCCAACAGATTCTACTGACCGCTTTGTGGCGGATGGTATCAGCGATCCAAAGCTTGTCGATTTCGGC
AAGCTCATCATGGCCACCTACGGCCAAGGAGCCAATGATGCCGCCCAACTCGGTGAAGTGCGAGTCGAGTACACCGTGCA
GCTCAAGAACAGAACTGGCTCAACCAGCGACGCCCAGATTGGGCAGTTCGCAGGTGTTAAGGACGGACCCAGGCTGGTTT
CATGGTCCAAGACCAAGGGGACAGCTGGGTGGGAGCACGATTGTCATTTTCTCGGAACCGGAAACTTCTCGTTGACATTGT
TCTACGAGAAGGCGCCGGTCTCGGGGCTAGAAAACGCAGACGCCTCTGACTTCTCGGTCCTGGGAGAAGCCGCAGCAGG
TAGTGTCCAATGGGCAGGAGTGAAGGTAGCAGAAAGGGGACAAGGCGTGAAAATGGTCACAACTGAGGAGCAGCCAAAG
GGTAAATTGCAAGCACTCAGAATTTAGAAGCGGCCGCGACTCTAGATCATAATCAGCCATACCACATTTGTAGAGGTTTTAC
TTGCTTTAAAAAACCTCCCACACCTCCCCCTGAACCTGAAACATAAAATGAATGCAATTGTTGTTGTTAACTTGTTTATTGCA
GCTTATAATGGTTACAAATAAAGCAATAGCATCACAAATTTCACAAATAAAGCATTTTTTTCACTGCATTCTAGTTGTGGTTTG
TCCAAACTCATCAATGTATCTTAAGCTTGGCGCGCCGTACGCGTATCGATAAGCTTAAGATACATGATGAGTTTGGACAAAC
CACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTATAAGCTGCAA
TAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAGGTTCAGGGGGAAGTGTGGGAGGTTTTTTAAAGCAAGTAAA
ACCTCTACAAATGTGGTATGGCTGATTATGATCTAGAGTCGCGGCCGCTACAGGAACAGGTGGTGGCGGCCCTCGGTGCG
CTCGTACTGCTCCACGATGGTGTAATCCTCGTTGTGGGAGGTGATGTCCAGCTTGGAGTCCACGTAGTAGTAGCCGGGCA
GCTGCACGGGCTTCTTGGCCATGTAGATGGACTTGAACTCCACCAGGTAGTGGCCGCCGTCCTTCAGCTTCAGGGCCTTG
TGGATCTCGCCCTTCAGCACGCCGTCGCGGGGGTACAGGCGCTCGGTGGAGGCCTCCCAGCCCATGGTCTTCTTCTGCA
TTACGGGGCCGTCGGAGGGGAAGTTCACGCCGATGAACTTCACCTTGTAGATGAAGCAGCCGTCCTGCAGGGAGGAGTC
CTGGGTCACGGTCACCACGCCGCCGTCCTCGAAGTTCATCACGCGCTCCCACTTGAAGCCCTCGGGGAAGGACAGCTTCT
TGTAGTCGGGGATGTCGGCGGGGTGCTTCACGTACACCTTGGAGCCGTACTGGAACTGGGGGGACAGGATGTCCCAGGC
GAAGGGCAGGGGGCCGCCCTTGGTCACCTTCAGCTTCACGGTGTTGTGGCCCTCGTAGGGGCGGCCCTCGCCCTCGCCC
TCGATCTCGAACTCGTGGCCGTTCACGGTGCCCTCCATGCGCACCTTGAAGCGCATGAACTCCTTGATGACGTTCTTGGAG
GAGCGCACCATGGTGGCGACCGGTGGATCCCGGGCCCGCGGTACCGTCGACTCTAGCGGTACCCCGATTGTTTAGCTTG
TTCAGCTGCGCTTGTTTATTTGCTTAGCTTTCGCTTAGCGACGTGTTCACTTTGCTTGTTTGAATTGAATTGTCGCTCCGTAG
ACGAAGCGCCTCTATTTATACTCCGGCGGTCGAGGGTTCGAAATCGATAAGCTTGGATCCTAATTGAATTAGCTCTAATTGA
ATTAGTCTCTAATGAATAATCCCCGCATTGTGGGAACCGTGCGATCAAACAAACGCGAGATACCGGAAGTACTGAAAAACA
GTCGCTCCAGGCCAGTGGGAACATCGATGTTTTGTTTTGACGGACCCCTTACTCTCGTCTCATATAAACCGAAGCCAGCTA
AGATGGTATACTTATTATCATCTTGTGATGAGGATGCTTCTATCAACGAAAGTACCGGTAAACCGCAAATGGTTATGTATTAT
AATCAAACTAAAGGCGGAGTGGACACGCTAGACCAAATGTGTTCTGTGATGACCTGCAGTAGGAAGACGAATAGGTGGCCT
ATGGCATTATTGTACGGAATGATAAACATTGCCTGCATAAATTCTTTTATTATATACAGCCATAATGTCAGTAGCAAGGGAGA
AAAGGTTCAAAGTCGCAAAAAATTTATGAGAAACCTTTACATGAGCCTGACGTCATCGTTTATGCGTAAGCGTTTAGAAGCT
CCTACTTTGAAGAGATATTTGCGCGATAATATCTCTAATATTTTGCCAAATGAAGTGCCTGGTACATCAGATGACAGTACTGA
AGAGCCAGTAATGAAAAAACGTACTTACTGTACTTACTGCCCCTCTAAAATAAGGCGAAAGGCAAATGCATCGTGCAAAAAA
TGCAAAAAAGTTATTTGTCGAGAGCATAATATTGATATGTGCCAAAGTTGTTTCTGACTGACTAATAAGTATAATTTGTTTCTA
TTATGTATAAGTTAAGCTAATTACTTATTTTATAATACAACATGACTGTTTTTAAAGTACAAAATAAGTTTATTTTTGTAAAAGA
GAGAATGTTTAAAAGTTTTGTTACTTTATAGAAGAAATTTTGAGTTTTTGTTTTTTTTTAATAAATAAATAAACATAAATAAATTG
TTTGTTGAATTTATTATTAGTATGTAAGTGTAAATATAATAAAACTTAATATCTATTCAAATTAATAAATAAACCTCGATATACA
GACCGATAAAACACATGCGTCAATTTTACGCATGATTATCTTTAACGTACGTCACAATATGATTATCTTTCTAGGGTTAAATAA
TAGTTTCTAATTTTTTTATTATTCAGCCTGCTGTCGTGAATACCGTATATCTCAACGCTGTCTGTGAGATTGTCGTATTCTAGC
CTTTTTAGTTTTTCGCTCATCGACTTGATATTGTCCGACACATTTTCGTCGATTTGCGTTTTGATCAAAGACTTGAGCAGAGA
CACGTTAATCAACTGTTCAAATTGATCCATATTAACGATATCAACCCGATGCGTATATGGTGCGTAAAATATATTTTTTAACCC
TCTTATACTTTGCACTCTGCGTTAATACGCGTTCGTGTACAGACGTAATCATGTTTTCTTTTTTGGATAAAACTCCTACTGAGT
TTGACCTCATATTAGACCCTCACAAGTTGCAAAACGTGGCATTTTTTACCAATGAAGAATTTAAAGTTATTTTAAAAAATTTCA
TCACAGATTTAAAGAAGAACCAAAAATTAAATTATTTAATCGACCAGTTAATCAACGTGTACACAGAGCGCAAAAAACACGCA
GCCCGACGTGTTGGCTAAAATTATTAAATCAACTTGTGTTATAGTCACGATTTGCCGTCCAACGTGTTCCTCAAAAAGTTGAA
GACCAACAAGTTTACGGACACTAGTTAATTATTTGATTTTGCCCCACTTCATTTTGTGGGATCACAATTTTGTTATATTTTAAA
CAAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCA
CATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGG
CGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATC
TGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCC
GGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCG
CGA

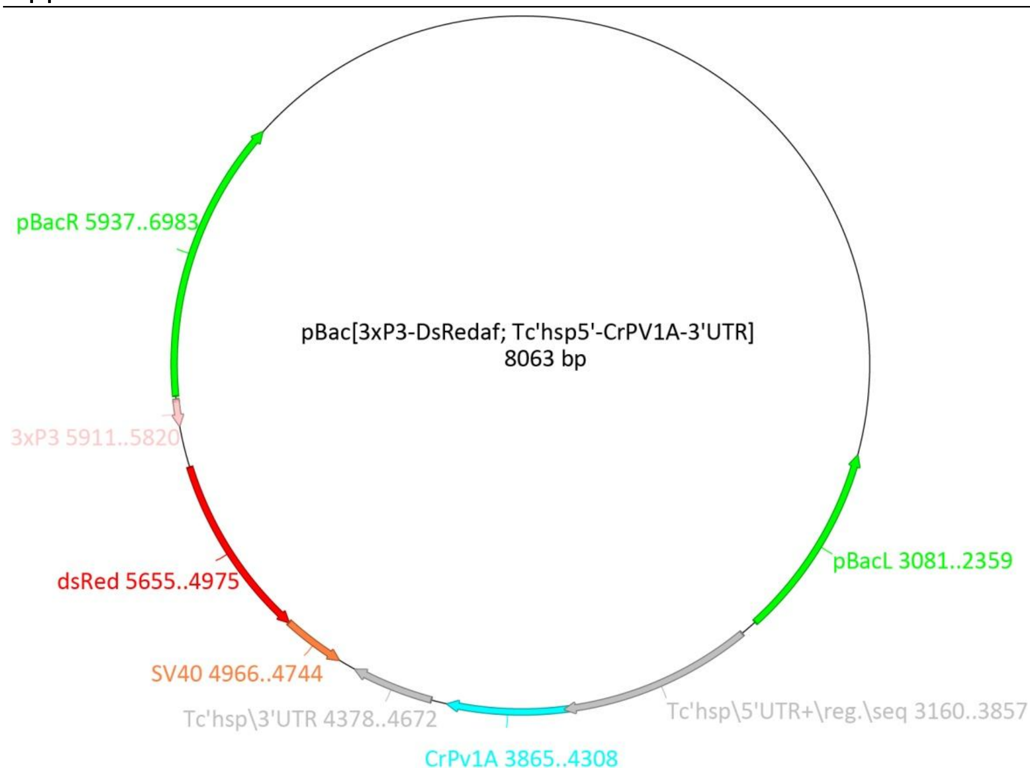


>pBac6XP3-ECFP;αTub1-3’UTRdcr2-intron-3’UTRdcr2-SV40

GACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTT
TCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCT
GATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCA
TTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGT
TACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTA
AAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGA
ATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCA
TAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACA
ACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACC
ACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAA
TAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAAT
CTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATC
TACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGG
TAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATC
CTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAG
GATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTT
GCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGT
GTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGC
TGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCT
GAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCATTGA
GAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACG
AGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTG
TGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCC
TTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTC
GCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCC
CCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATT
AATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCG
GATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACG
CTCGCGCGACTTGGTTTGCCATTCTTTAGCGCGCGTCGCGTCACACAGCTTGGCCACAATGTGGTTTTTGTCAAACGAAGA
TTCTATGACGTGTTTAAAGTTTAGGTCGAGTAAAGCGCAAATCTTTTTTAACCCTAGAAAGATAGTCTGCGTAAAATTGACGC

ATGCATTCTTGAAATATTGCTCTCTCTTTCTAAATAGCGCGAATCCGTCGCTGTGCATTTAGGACATCTCAGTCGCCGCTTG
GAGCTCCCGTGAGGCGTGCTTGTCAATGCGGTAAGTGTCACTGATTTTGAACTATAACGACCGCGTGAGTCAAAATGACGC
ATGATTATCTTTTACGTGACTTTTAAGATTTAACTCATACGATAATTATATTGTTATTTCATGTTCTACTTACGTGATAACTTAT
TATATATATATTTTCTTGTTATAGATATCGTGACTAATATATAATAAAATGGGTAGTTCTTTAGACGATGAGCATATCCTCTCT
GCTCTTCTGCAAAGCGATGACGAGCTTGTTGGTGAGGATTCTGACAGTGAAATATCAGATCACGTAAGTGAAGATGACGTC
CAGAGCGATACAGAAGAAGCGTTTATAGATGAGGTACATGAAGTGCAGCCAACGTCAAGCGGTAGTGAAATATTAGACGAA
CAAAATGTTATTGAACAACCAGGTTCTTCATTGGCTTCTAACAGAATCTTGACCTTGCCACAGAGGACTATTAGAGGTAAGA
ATAAACATTGTTGGTCAACTTCAAAGTCCACGAGGCGTAGCCGAGTCTCTGCACTGAACATTGTCAGATCTAAGATAGGGC
CCCTTAAGGTACTATGGTTGCTTTGACGTATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGC
GCCATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTATTACGCCAGCTGGCGAAA
GGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGC
CAAGCTGGCCGGCCTAGGCGCGCCAAGCTCTAGTCGCGCTCGCGCGACTGACGGTCGTAAGCACCCGCGTACGTGTCCA
CCCCGGTCACAACCCCTTGTGTCATGTCGGCGACCCTACGCCCCCAACTGAGAGAACTCAAAGGTTACCCCAGTTGGGGC
ACTACTCCCGAAAACCGCTTCTGACCTGGGAAAACGTGAAGCCCCGGGGCATCCGCTGAGGGTTGCCGCCGGGGCTTCG
GTGTGTCCGTCAGTACTAGAGCTCTCGAGACCTCACACTTGCCGTAAATGGAGGTGATATCACAAATCCCTACTAAACGTG
GTATTCGCGGCATGACTCATAGTTTATTTGAAATCATTTTAACCCTAATCTTTTAATCAATAAACTGAATTTTTCTCAAGTTAAT
GCCTTTTCTTGGTTTGGAAATAAGTGTGTTATTAATAGCTTTAACTGTCTACAATGCTGAAGAACCAGCAAGTTTATTTTGGG
GTCAAAGAAAGCACTTGAAAAGGGTGCCAGATGTCTATGTATCTCCCGTGAAACTATTTTAAAAGGAGTTTATCACACGTTTT
CATATCTATTGTCTTATGAAAGAAGGGAAAAGTAGAGTTTTCTTGAAGAAACTGTCCCAAAATTAATATTATTGAACAAAAAAT
TCTGAGAAATTCACACTGCAGTGAACGGTTATGATGGTGCAACGACCGGGACCTCAGCTGTATAAAAAATCTGAAAATAATT
GATAATGGTCGAAGACCATAAGGAAAAATAATTCGAAAATTAAAGGCAAAGTATGAAAATGCTGGAAATAAATCGGGAAACA
CCGAATTTTTGGTCACAGGAACCAACGAATTTTTTTTTGAATATTGATTTGAAAAGAGATCGAGAACAAGGATAACAATCTTC
CGTTAGAGACCGTTGGTACCGACATGGCGGGGAGCCAATCAGAAGTCTGGAAATCGGGCGTTGGTTGCTGACGTCAACAA
GCTTATAAATAGCAACGACAGTTGAAAATCGAATCAAAGTCGTTTGGAAAAAGCCAGAGCTTGTATTTCCGAAGCGTACTCC
CGTTTTTCTGCTCTTTTGTGGTGTAATTTGTAAAACTCAACTACCAAGCTAGTAACTTGAAACAGTTTTAATTTGAAAACGAAT
TATTTTTGGAACCACCTAATGACAAATTAAAGGTTGGCTGTAATTTAAAAATTCTCCTACTTAATTATCAGCGTGAGTTAAATT
CACCATTTTTGTGATAGGTATCAAAGCGAATGAATTTAACTGACGGTATATAGTTGAACAATGGAAAACAGCATTTTTCAAAC
AATCCTAGCGAGTAACTGAATCAATATAAGCGCATTGTGACTTCGTGCGTTAATACCAGAGAGAAGATCGTGTTACCGAACG
CCCAAGGTAAACAAACACCCGGCCCTGGATGGTTGCGGCTAACACAACCCCTGCGTTTGGCTTCTTTCACTCGCTACTAAT
ATTTGCCCAGATTTTTGGATAAGTGGGTTTGCACCCACATTGCCAAGAGCAATACCTCACATAACGAGATTATTCACTTTCG
GTGCGAATGAGTCATGGAAAAAGTGAAACAAGATTGCGTTTTAAATTTTTGCTGGAGGGTTTTTTCTGTTAATTACACGCCAA
ATGTTAAATTTGTTCAATGCAGATGTCATTACGGATCGTTTGTAAATTTGTTTCTAAAACACATCTGGCAAATGCAGCCTACA
TAAAACTTGTTAGAAAACAAATCTAATATTTATTAATTCTCAATTTTTCATTGACCAAGCAGATAAGAGTACCACATGCAGAAC
GGAAACCGAACGTTAAAACACTGATAATATGTTGGTTGTTGGTAGCCAATCCAGTTTACGACCAGGATTAGAAGTTATCGAC
CTTTTATGGTTTAATCATTGCTAACTCTAAAACGTTCCAACTAATTCCTTATAAATCATTTCATGTAAATTTAACTCACAATTAA
ACTTGACCGATTTTAATGCAAGTTATTGCGTCAACAGTTAAAATATCGCACAAATATTCATGTAGCGATTTTCGATTGAAGGG
TCATGGTTGGGTTATCTTCAGTCGGATTGAAAAGCTCAAAGAGGCACTGTAATTACCATTCTGTTTTCAGTCGGCTTTAGGC
GGATTTCCGCTTGATAACAATATCACTAACACAGCGATTGACATGTTTGCTTACACTCCTCGATGATTTCCTAATATTGGGTT
ATACTTTTTAGATGTAGCGGCCGAGAAAACGGAGAAAGCTCTGATCGCCGGTATCGAGCATTTCGATCCAAGTATTAACGC
ACGAAGTCACAATGCGCTTATATTGATTCAGTTACTCGCTAGGATTGTTTGAAAAATGCTGTTTTCCATTGTTCAACTATATA
CCGTCAGTTAAATTCATTCGCTTTGATACCTATCACAAAAATGGTGAATTTAACTCACGCTGATAATTAAGTAGGAGAATTTTT
AAATTACAGCCAACCTTTAATTTGTCATTAGGTGGTTCCAAAAATAATTCGTTTTCAAATTAAAACTGTTTCAAGTTACCTGAT
GAGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAAC
CATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAGGTTCAGGGGGAAGTGTGGGAGGTTT
TTTAAAGCAAGTAAAACCTCTACAAATGTGGTATGGCTGATTATGATCTAGAGTCGCGGCCGCTTTACTTGTACAGCTCGTC
CATGCCGAGAGTGATCCCGGCGGCGGTCACGAACTCCAGCAGGACCATGTGATCGCGCTTCTCGTTGGGGTCTTTGCTCA
GGGCGGACTGGGTGCTCAGGTAGTGGTTGTCGGGCAGCAGCACGGGGCCGTCGCCGATGGGGGTGTTCTGCTGGTAGT
GGTCGGCGAGCTGCACGCTGCCGTCCTCGATGTTGTGGCGGATCTTGAAGTTGGCCTTGATGCCGTTCTTCTGCTTGTCG
GCGGTGATATAGACGTTGTGGCTGATGTAGTTGTACTCCAGCTTGTGCCCCAGGATGTTGCCGTCCTCCTTGAAGTCGATG
CCCTTCAGCTCGATGCGGTTCACCAGGGTGTCGCCCTCGAACTTCACCTCGGCGCGGGTCTTGTAGTTGCCGTCGTCCTT
GAAGAAGATGGTGCGCTCCTGGACGTAGCCTTCGGGCATGGCGGACTTGAAGAAGTCGTGCTGCTTCATGTGGTCGGGGT

AGCGGCTGAAGCACTGCACGCCCCAGGTCAGGGTGGTCACGAGGGTGGGCCAGGGCACGGGCAGCTTGCCGGTGGTGC
AGATGAACTTCAGGGTCAGCTTGCCGTAGGTGGCATCGCCCTCGCCCTCGCCGGACACGCTGAACTTGTGGCCGTTTACG
TCGCCGTCCAGCTCGACCAGGATGGGCACCACCCCGGTGAACAGCTCCTCGCCCTTGCTCACCATGGTGGCGACCGGTA
CCACTTTGAATTCACTAGTAAATAATTCACTCAACTTTGTTAAAGTCGCTTTGAAAATTTGCTTTGAGTCGCTTGCTTAGCTTT
GTTGCTTTCGCTTCGCTTGATTCAAATTCACTGACAACGCGCCGCGAGACCGCGCTTATATATGAAACGGATTAATTGAATT
AGCTCTAATTGAATTAGTCTCTAATTGAATTAGATCTTAATTGAATTAGCTCTAATTGAATTAGTCTCTAATTGAATTAGATCGT
CGACGTCCCATGGAACCATTGTGGGAACCGTGCGATCAAACAAACGCGAGATACCGGAAGTACTGAAAAACAGTCGCTCC
AGGCCAGTGGGAACATCGATGTTTTGTTTTGACGGACCCCTTACTCTCGTCTCATATAAACCGAAGCCAGCTAAGATGGTAT
ACTTATTATCATCTTGTGATGAGGATGCTTCTATCAACGAAAGTACCGGTAAACCGCAAATGGTTATGTATTATAATCAAACT
AAAGGCGGAGTGGACACGCTAGACCAAATGTGTTCTGTGATGACCTGCAGTAGGAAGACGAATAGGTGGCCTATGGCATT
ATTGTACGGAATGATAAACATTGCCTGCATAAATTCTTTTATTATATACAGCCATAATGTCAGTAGCAAGGGAGAAAAGGTTC
AAAGTCGCAAAAAATTTATGAGAAACCTTTACATGAGCCTGACGTCATCGTTTATGCGTAAGCGTTTAGAAGCTCCTACTTTG
AAGAGATATTTGCGCGATAATATCTCTAATATTTTGCCAAATGAAGTGCCTGGTACATCAGATGACAGTACTGAAGAGCCAG
TAATGAAAAAACGTACTTACTGTACTTACTGCCCCTCTAAAATAAGGCGAAAGGCAAATGCATCGTGCAAAAAATGCAAAAA
AGTTATTTGTCGAGAGCATAATATTGATATGTGCCAAAGTTGTTTCTGACTGACTAATAAGTATAATTTGTTTCTATTATGTAT
AAGTTAAGCTAATTACTTATTTTATAATACAACATGACTGTTTTTAAAGTACAAAATAAGTTTATTTTTGTAAAAGAGAGAATGT
TTAAAAGTTTTGTTACTTTATAGAAGAAATTTTGAGTTTTTGTTTTTTTTTAATAAATAAATAAACATAAATAAATTGTTTGTTGA
ATTTATTATTAGTATGTAAGTGTAAATATAATAAAACTTAATATCTATTCAAATTAATAAATAAACCTCGATATACAGACCGATA
AAACACATGCGTCAATTTTACGCATGATTATCTTTAACGTACGTCACAATATGATTATCTTTCTAGGGTTAAATAATAGTTTCT
AATTTTTTTATTATTCAGCCTGCTGTCGTGAATACCGTATATCTCAACGCTGTCTGTGAGATTGTCGTATTCTAGCCTTTTTAG
TTTTTCGCTCATCGACTTGATATTGTCCGACACATTTTCGTCGATTTGCGTTTTGATCAAAGACTTGAGCAGAGACACGTTAA
TCAACTGTTCAAATTGATCCATATTAACGATATCAACCCGATGCGTATATGGTGCGTAAAATATATTTTTTAACCCTCTTATAC
TTTGCACTCTGCGTTAATACGCGTTCGTGTACAGACGTAATCATGTTTTCTTTTTTGGATAAAACTCCTACTGAGTTTGACCT
CATATTAGACCCTCACAAGTTGCAAAACGTGGCATTTTTTACCAATGAAGAATTTAAAGTTATTTTAAAAAATTTCATCACAGA
TTTAAAGAAGAACCAAAAATTAAATTATTTAATCGACCAGTTAATCAACGTGTACACAGAGCGCAAAAAACACGCAGCCCGA
CGTGTTGGCTAAAATTATTAAATCAACTTGTGTTATAGTCACGATTTGCCGTCCAACGTGTTCCTCAAAAAGTTGAAGACCAA
CAAGTTTACGGACACTAGTTAATTATTTGATTTTGCCCCACTTCATTTTGTGGGATCACAATTTTGTTATATTTTAAACAAAGC
TTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCC
CCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATG
GCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATATGGTGCACTCTCAGTACAATCTGCTCT
GATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCAT
CCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGA



>pBac3xP3-DsRed;Tc'hsp5'-CrPV1A-3'UTR

GACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTT
TCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCT
GATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCA
TTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGT
TACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTA
AAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGA
ATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCA
TAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACA
ACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACC
ACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAA
TAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAAT
CTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATC
TACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGG
TAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGATC
CTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAG
GATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTT
GCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTCCTTCTAGT
GTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGC
TGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCT
GAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCATTGA
GAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACG
AGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTG
TGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCC
TTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTC
GCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCC
CCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATT
AATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCG
GATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACG

CTCGCGCGACTTGGTTTGCCATTCTTTAGCGCGCGTCGCGTCACACAGCTTGGCCACAATGTGGTTTTTGTCAAACGAAGA
TTCTATGACGTGTTTAAAGTTTAGGTCGAGTAAAGCGCAAATCTTTTTTAACCCTAGAAAGATAGTCTGCGTAAAATTGACGC
ATGCATTCTTGAAATATTGCTCTCTCTTTCTAAATAGCGCGAATCCGTCGCTGTGCATTTAGGACATCTCAGTCGCCGCTTG
GAGCTCCCGTGAGGCGTGCTTGTCAATGCGGTAAGTGTCACTGATTTTGAACTATAACGACCGCGTGAGTCAAAATGACGC
ATGATTATCTTTTACGTGACTTTTAAGATTTAACTCATACGATAATTATATTGTTATTTCATGTTCTACTTACGTGATAACTTAT
TATATATATATTTTCTTGTTATAGATATCGTGACTAATATATAATAAAATGGGTAGTTCTTTAGACGATGAGCATATCCTCTCT
GCTCTTCTGCAAAGCGATGACGAGCTTGTTGGTGAGGATTCTGACAGTGAAATATCAGATCACGTAAGTGAAGATGACGTC
CAGAGCGATACAGAAGAAGCGTTTATAGATGAGGTACATGAAGTGCAGCCAACGTCAAGCGGTAGTGAAATATTAGACGAA
CAAAATGTTATTGAACAACCAGGTTCTTCATTGGCTTCTAACAGAATCTTGACCTTGCCACAGAGGACTATTAGAGGTAAGA
ATAAACATTGTTGGTCAACTTCAAAGTCCACGAGGCGTAGCCGAGTCTCTGCACTGAACATTGTCAGATCCGAGATCGGCC
GGCCGAATTCGAATGGCCATGGGACGTCGACCTGAGGTAATTATAACCCGGGCCCTATATATGGATCCCAACCTTCAATAA
AAAATTGTTCTTTATTTATTATTAATCACTATTATTTAGGCATCTTCTGCAAAAGTAATTGATTATAAACGATAAAAGTAAAAAA
AAGGATTAATCTAAAACATTTTACTTAAAGACCTATTAATAAGTTTAAAAAGAAAAAAAATAGGAAAATTGTTTTATTAAAATTG
CTATGACAATCCAACTTTGGCATATGTATATGAAATCAAAACAATTCACTACTCATTCATAATATTGCCGAACTTTACCAAATT
AAAAATAAACATGAAATAGTCAACAACTCATTCGTTATGTAACGTTTTTTTTTTAAATGCATTAATATATAATGATTGTTTTTATT
TTGGAAGTTGAAATAAATTAGGGCACAACCAGTTTCCACTATTCTCCTTTATCTTTACGGAAATAGCATGAATAATACCGAAA
AGGCCGTTTTAAAAATAAAAATTCGAGAAAATTCAAACGCAAATCAATGGAAACTTCGAGATCATTCGGTGAAGTTCGAGAC
GAATTCCCCGTTTCATATATAAGCGCGGTCTCGCGGCGCGTTGTCAGTGAATTTGAATCAAGCGAAGCGAAAGCAACAAAG
CTAAGCAAGCGACTCAAAGCAAATTTTCAAAGCGACTTTAACAAAGTTGAGTGAATTATTTACTAGTGAATTCAAAGTAAATC
GAAAAGTGTATCTAGAGGTACCATGTCTTTTCAACAAACAAACAACAACGCAACCAACAACATCAACTCCCTTGAGGAGCTT
GCTGCTCAAGAACTAATAGCAGCACAATTTGAAGGAAATCTTGATGGTTTCTTTTGCACTTTTTATGTGCAGTCCAAACCACA
ACTATTGGACTTAGAGAGTGAATGTTATTGTATGGATGATTTTGATTGTGGGTGTGATAGGATCAAGAGAGAAGAAGAATTA
CGTAAACTGATTTTCTTAACATCGGACGTTTATGGATATAACTTTGAAGAGTGGAAAGGATTAGTTTGGAAATTTGTTCAAAA
TTATTGCCCAGAACATCGATATGGATCAACTTTTGGTAATGGATTATTAATTGTGAGTCCCCGTTTCTTTATGGATCATCTTG
ACTGGTTTCAGCAATGGAAACTTGTTTCAAGTAATGATGAATGCAGAGCCTTCAAGCGGCCGCCTGCAGCTGGCGCCATCG
ATACGCGTACGTCGCGACCGCGGACATGTACAGAGCTCGAGATGATCGGTTTGTGTTCCTACTTAGTTAATTGTGATAGTTT
ATTTTTGTAAATAATAGTACGTAAAGTTACTTTAGTTATAATAAAGTAGATAGTGACTGCATTAAGTTTAAAGACTGATTTTTGT
AGATTATTTAAAAATTTTTAATAAATTATTTAATTTAAAGATAAAAAGTTGTTTCATTTTAAAATAATAAAAAACTTACCGCATTA
GTCGTGGCCTTGGCAGTATTAAGCGCAAACGGGCTCTCCTTCGTATGCCCATCAGGAATATAGAAAAAAACAACTGGCTGC
TCACTAGTGGCCACGTGGGCCGTGCACCTTAAGCTTGGCGCGCCGTACGCGTATCGATAAGCTTAAGATACATGATGAGTT
TGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTATTTGTAACCATTAT
AAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAGGTTCAGGGGGAAGTGTGGGAGGTTTTTTAAA
GCAAGTAAAACCTCTACAAATGTGGTATGGCTGATTATGATCTAGAGTCGCGGCCGCTACAGGAACAGGTGGTGGCGGCC
CTCGGTGCGCTCGTACTGCTCCACGATGGTGTAGTCCTCGTTGTGGGAGGTGATGTCCAGCTTGGAGTCCACGTAGTAGT
AGCCGGGCAGCTGCACGGGCTTCTTGGCCATGTAGATGGACTTGAACTCCACCAGGTAGTGGCCGCCGTCCTTCAGCTTC
AGGGCCTTGTGGATCTCGCCCTTCAGCACGCCGTCGCGGGGGTACAGGCGCTCGGTGGAGGCCTCCCAGCCCATGGTCT
TCTTCTGCATTACGGGGCCGTCGGAGGGGAAGTTCACGCCGATGAACTTCACCTTGTAGATGAAGCAGCCGTCCTGCAGG
GAGGAGTCCTGGGTCACGGTCACCACGCCGCCGTCCTCGAAGTTCATCACGCGCTCCCACTTGAAGCCCTCGGGGAAGG
ACAGCTTCTTGTAGTCGGGGATGTCGGCGGGGTGCTTCACGTACACCTTGGAGCCGTACTGGAACTGGGGGGACAGGAT
GTCCCAGGCGAAGGGCAGGGGGCCGCCCTTGGTCACCTTCAGCTTCACGGTGTTGTGGCCCTCGTAGGGGCGGCCCTCG
CCCTCGCCCTCGATCTCGAACTCGTGGCCGTTCACGGTGCCCTCCATGCGCACCTTGAAGCGCATGAACTCCTTGATGAC
GTTCTTGGAGGAGCGCACCATGGTGGCGACCGGTGGATCCCGGGCCCGCGGTACCGTCGACTCTAGCGGTACCCCGATT
GTTTAGCTTGTTCAGCTGCGCTTGTTTATTTGCTTAGCTTTCGCTTAGCGACGTGTTCACTTTGCTTGTTTGAATTGAATTGT
CGCTCCGTAGACGAAGCGCCTCTATTTATACTCCGGCGGTCGAGGGTTCGAAATCGATAAGCTTGGATCCTAATTGAATTA
GCTCTAATTGAATTAGTCTCTAATTGAATTAGATCCCCGGGCGAGCTCGAATTAACCATTGTGGGAACCGTGCGATCAAACA
AACGCGAGATACCGGAAGTACTGAAAAACAGTCGCTCCAGGCCAGTGGGAACATCGATGTTTTGTTTTGACGGACCCCTTA
CTCTCGTCTCATATAAACCGAAGCCAGCTAAGATGGTATACTTATTATCATCTTGTGATGAGGATGCTTCTATCAACGAAAGT
ACCGGTAAACCGCAAATGGTTATGTATTATAATCAAACTAAAGGCGGAGTGGACACGCTAGACCAAATGTGTTCTGTGATGA
CCTGCAGTAGGAAGACGAATAGGTGGCCTATGGCATTATTGTACGGAATGATAAACATTGCCTGCATAAATTCTTTTATTATA
TACAGCCATAATGTCAGTAGCAAGGGAGAAAAGGTTCAAAGTCGCAAAAAATTTATGAGAAACCTTTACATGAGCCTGACGT
CATCGTTTATGCGTAAGCGTTTAGAAGCTCCTACTTTGAAGAGATATTTGCGCGATAATATCTCTAATATTTTGCCAAATGAA

GTGCCTGGTACATCAGATGACAGTACTGAAGAGCCAGTAATGAAAAAACGTACTTACTGTACTTACTGCCCCTCTAAAATAA
GGCGAAAGGCAAATGCATCGTGCAAAAAATGCAAAAAAGTTATTTGTCGAGAGCATAATATTGATATGTGCCAAAGTTGTTT
CTGACTGACTAATAAGTATAATTTGTTTCTATTATGTATAAGTTAAGCTAATTACTTATTTTATAATACAACATGACTGTTTTTA
AAGTACAAAATAAGTTTATTTTTGTAAAAGAGAGAATGTTTAAAAGTTTTGTTACTTTATAGAAGAAATTTTGAGTTTTTGTTTT
TTTTTAATAAATAAATAAACATAAATAAATTGTTTGTTGAATTTATTATTAGTATGTAAGTGTAAATATAATAAAACTTAATATCT
ATTCAAATTAATAAATAAACCTCGATATACAGACCGATAAAACACATGCGTCAATTTTACGCATGATTATCTTTAACGTACGTC
ACAATATGATTATCTTTCTAGGGTTAAATAATAGTTTCTAATTTTTTTATTATTCAGCCTGCTGTCGTGAATACCGTATATCTCA
ACGCTGTCTGTGAGATTGTCGTATTCTAGCCTTTTTAGTTTTTCGCTCATCGACTTGATATTGTCCGACACATTTTCGTCGAT
TTGCGTTTTGATCAAAGACTTGAGCAGAGACACGTTAATCAACTGTTCAAATTGATCCATATTAACGATATCAACCCGATGCG
TATATGGTGCGTAAAATATATTTTTTAACCCTCTTATACTTTGCACTCTGCGTTAATACGCGTTCGTGTACAGACGTAATCAT
GTTTTCTTTTTTGGATAAAACTCCTACTGAGTTTGACCTCATATTAGACCCTCACAAGTTGCAAAACGTGGCATTTTTTACCAA
TGAAGAATTTAAAGTTATTTTAAAAAATTTCATCACAGATTTAAAGAAGAACCAAAAATTAAATTATTTAATCGACCAGTTAATC
AACGTGTACACAGAGCGCAAAAAACACGCAGCCCGACGTGTTGGCTAAAATTATTAAATCAACTTGTGTTATAGTCACGATT
TGCCGTCCAACGTGTTCCTCAAAAAGTTGAAGACCAACAAGTTTACGGACACTAGTTAATTATTTGATTTTGCCCCACTTCAT
TTTGTGGGATCACAATTTTGTTATATTTTAAACAAAGCTTGGCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCC
TGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATC
GCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTT
CACACCGCATATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCC
GCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATGT
GTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGA