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The Role of *cort* and Anaphase Promoting Complex/Cyclosome (APC/C) in *Drosophila* Sex Determination and Meiosis

By

Abuzar Sikandar Malik

A Thesis

Submitted to the Faculty of Graduate Studies

through the Department of Integrative Biology and the Department of Biomedical Sciences

in Partial Fulfillment of the Requirements for

the Degree of Master of Science

at the University of Windsor

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ABSTRACT

The E3 ubiquitin ligase, APC/C, is essential for the completion cell cycle; along with its co-activators it allows mitotic exit and maintenance of G1. APC/C marks various substrates with ubiquitin chains; marked substrates are subsequently destroyed via the 26S proteasome pathway. Cort is a *Drosophila* female meiosis specific activator of APC/C. Cort works within meiosis in conjunction with Fzy to mediate Securin and cyclin destruction. A C-terminal IR-tail motif and a N-terminal C-box support Cort-APC/C interaction, whereas short motifs like D-box and KEN-box on the target protein impart substrate recognition to Cort. *Cort* expression is tightly controlled in the female germline; and our lab found that misexpression of *cort* outside of this window results in a unique phenotype where females are transformed to male-like individuals. We provide evidence that this sex transformation is due to the actions of APC/C^{Cort} on the sex determination factor, Transformer (Tra). Genetic epistasis analysis showed that *cort* is partially epistatic to *tra*. Western blot analysis shows that in the presence of Cort, Tra^f (female isoform of Tra protein) levels go down significantly. This reduction of Tra^f levels is likely through APC/C^{Cort} mediated activity, as rendering APC/C ineffective via RNAi results in loss of sex transformation in Cort misexpressing individuals. Loss of maternal *cort* leads to reduced male viability and produce males with abnormal or missing genitalia and analia. This phenotype could potentially be a consequence of Tra^f weakly initiating the Sxl positive feedback loop in males. We hypothesize that maternal Cort mediated Tra^f destruction functions to safeguard the sexual morphology and viability of male progeny. We theorize that maternally deposited Cort is tasked with preventing untimely *Sxl* activation to ensure proper male development. This property of Tra could potentially have evolutionary implications for other Diptera species, where maternal Cort could target maternal Tra to stop male progeny from transforming into females.

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This effort would have been impossible without my loving parents. Their unrelenting support and affection from halfway across the world fuels my resolve. I extend my appreciation to my brothers and sister; their presence and companionship make all this tolerable.

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To the countless flies culled for this data

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CHAPTER I: INTRODUCTION AND BACKGROUND

The cell cycle

The eukaryotic cell cycle is the 4-stage process by which new cells are generated (Cooper, 2000). The 4 distinct phases of the cell cycle are as follows: the G1 phase, S phase, G2 phase and M phase. Commonly the G1, S and G2 phases are merged into the Interphase. The two gap or growth (G1/G2) phases are where the cell grows, carries out its regular functions and accumulates nutrients and energy in preparation for the S and M phase. The Synthesis (S) phase is where the DNA is synthesized or replicated in preparation for the division. M phase is either mitosis or meiosis. Mitosis itself is divided into different stages. Prophase is the initial stage where the chromosomes condense, and the spindle start to form. This is followed by prometaphase, where the nuclear envelope breaks down and the spindles microtubules seek out and bind to the kinetochores of the sister chromatids; in a "search and capture" mechanism. Following prometaphase the cell enters metaphase, where all sister chromatids are aligned on the metaphase plate (the cell equator) and connected by kinetochore microtubules to both poles of the spindle. Once an appropriate signal is received, the cycle transitions to anaphase; here the cohesin complex is cleaved by Separase and sister chromatids can now be separated and are pulled towards the cell poles, driven by the pulling forces of the microtubules. Telophase followed by cytokinesis marks the end of mitosis. These phases are defined by the formation of the new nuclear envelope around each new daughter nucleus and the distribution of cytoplasm, nutrients, and organelles between the new daughter cells. Some cells can enter a G_0 phase; this is a quiescence phase where a fully differentiated cell can remain indefinitely (in the case of neurons) or for an extended period of time (some kidney and liver cells); the cells continue to perform their tasks but do not transition into S-phase or M-phase and divide (Cooper, 2000).

Rigorous control over the initiation, inhibition, and progression of each stage of the cell cycle is imperative for cell survival and overall health of the organism. Several checkpoints exist under the control of various regulatory molecules, in both interphase and mitotic phase. M phase entry happens under the supervision of cyclin and cyclin-dependent kinases, specifically, Cyclin B which complexes with Cdk1 to form the M-phase promoting factor (MPF); permitting the cell

to transition into the M-phase. Departure from M-phase is managed by the APC/C via the destruction of cyclins and other substrates (Westendorf et al., 1989).

APC/C

The Anaphase Promoting Complex/Cyclosome (APC/C), is an E3 ubiquitin ligase that induces the destruction of various substrates during the metaphase-anaphase transition in mitosis and meiosis. The APC/C is a large multi-subunit complex consisting of <20 subunits, depending on organism (13 in *Drosophila*, Figure 1.1 and Table 1.1) (Pesin & Orr-Weaver, 2008; Primorac & Musacchio, 2013).

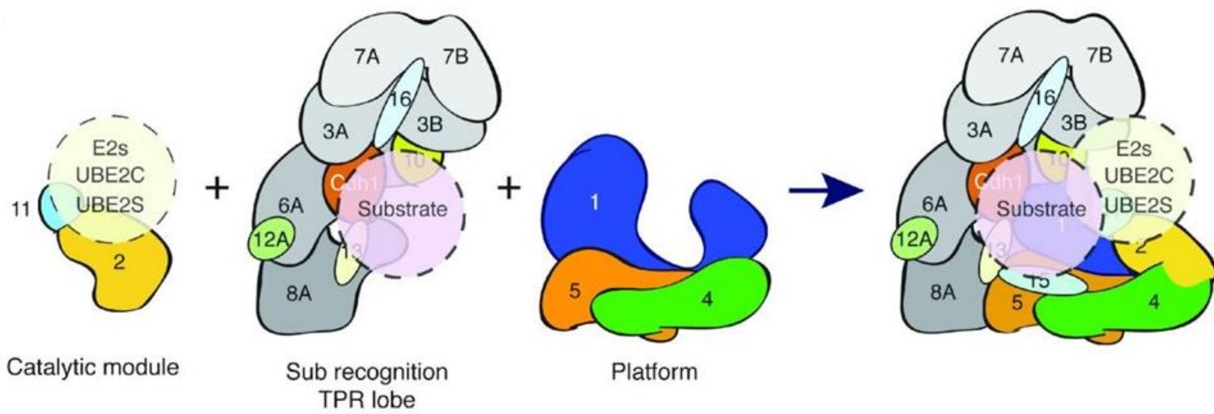

Figure 1.1. The three modules of the APC/C. The catalytic module (Apc2-Apc11), that interacts with E2s, the substrate recognition TPR lobe, and the scaffolding platform (Apc1-Apc4-Apc5). The substrate and E2s are positioned in or near the central cavity (Yamano, 2019).

The APC/C's interaction with the E2s is facilitated by the RING domains within APC/C. This brings the substrate (which is bound to recognition sites elsewhere on the APC/C) within close proximity of the E2. The ubiquitin chain is established by an E2, Ube2C. Meanwhile, the job of extending this chain is performed by a second E2, Ube2S (Yu et al., 1996). The APC/C behaves as a two-substrate enzyme, as it itself is not chemically engaged in ubiquitin transfer

but, instead, prompts the two substrates together (E2~Ub which transfers the Ub onto a target substrate), thereby "catalyzing" the ubiquitin transfer (Yamano, 2019) (Figure 1.2).

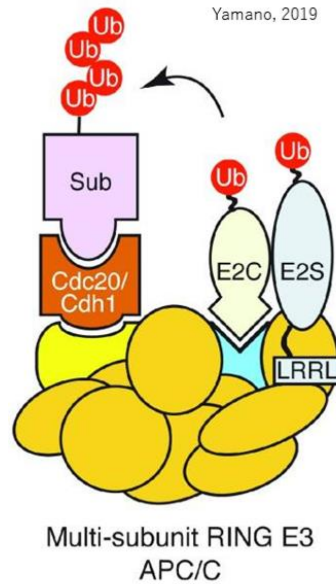

Figure 1.2. The APC/C is a multi-subunit cullin-RING E3 ubiquitin ligase that uses two E2 enzymes (Ube2C and Ube2S) that help achieve ubiquitination of traget (Yamano, 2019).

The length and topology of the ubiquitin chain conveys vital information to the 26S proteosome. Any ubiquitinated target is subsequently destroyed in a proteosome-dependent proteolysis (Peters, 2006; Lu et al., 2015). Structurally and functionally, the subunits of the APC/C can be grouped into three classes: the substrate recognition module, the scaffolding module, and the catalytic module (Figure 1.1). The substrate recognition and the catalytic modules contain most of the principal subunits of the APC/C, which is the reason for their high conservation among all types of creatures.

Most of the mass of the APC/C is sequestered in the scaffolding module. The primary job of the scaffolding module is mediating protein–protein interactions and the assembly of multi-protein complexes. The subunits of the catalytic module, APC2 and APC11, as the name implies, have been implicated in the catalysis and regulation of the APC/C (Primorac & Musacchio, 2013; Yamano, 2019).

APC10 together with the co-activators (Cdc20 and Cdh1; Fzy and Fzr in *Drosophila*) form the substrate recognition module. Substrate recognition is imperative and a prerequisite for ubiquitination catalysis. The WD40 β-propeller domains of these co-activators provide APC/C with its degron recognition. Cdc20 identifies substrates with a destruction box (D-box, RxxLxxxxN), while Cdh1 identifies the D-box and KEN box (KENxxxN) (Peters, 2006; Alfieri et al., 2017). Other motifs have also been identified (ABBA motif) or are still under investigation and are less characterized (CRY-box or the O-box) (Primorac & Musacchio, 2013; Yamano, 2019).

Vertebrate	*Drosophila*	Structural motif	Function
APC/C subunits			
APC1	Shattered	WD40	Scaffolding module
APC2	Morula	NTD	Catalytic module
APC3	Cdc27	TPR dimer	Scaffolding module
APC4	Unknown	WD40	Scaffolding module
APC5	Ida	NTD	Scaffolding module
APC6	Cdc16	TPR dimer	Scaffolding module
APC7	N/A	TPR dimer	Scaffolding module
APC8	Cdc23	TPR dimer	Scaffolding module
APC9	N/A		Stabilizes Cdc27
APC10	APC10	Doc homology	Degron recognition module
APC11	Lemming	β-strand	Catalytic module
APC12	N/A	Extended chain, short α-helix	Scaffolding module
APC13	APC13	Extended chain	Scaffolding module
APC15	Unknown	Extended chain and α-helix	Scaffolding module
APC16	Unknown	α-helix	Scaffolding module
Cdc20/Cdh1	Fzy/Fzr	NTD, WD40, IR tail	Degron recognition module
UbcH10	N/A	UBC domain	Catalytic module

Table 1.1. APC/C subunits and their structural motifs and functions. (adapted from Batiha (2013) and Alfieri et al., 2017)

The strength of the interaction between the substrate and the co-activator can be manipulated by environmental cues. For instance, the phosphorylation state near the degron sequence can impact the ubiquitination of the substrate. It is speculated that substrate-APC/C interactions that are too strong or too weak can also have a negative influence on ubiquitination.

Competitive inhibitors of the APC/C, like Mes1 and Acm1, work by having excessively strong affinity to APC/C (Yamano, 2019).

APC/C in mitosis

For any living thing, proper regulation of mitosis is imperative for development, cell proliferation and self-renewal. Losing domain over this aspect of cellular biology has dire consequences, chief among them is cancer, which is one of the leading causes of death in humans.

The temporal and spatial regulation of the APC/C is how mitosis progression is controlled and coordinated. Cyclin B/Cdk1 phosphorylates multiple APC/C subunits thereby granting Cdc20 to activate APC/C. APC/C^{Cdc20} acts by targeting multiple mitotic cyclins for destruction which leads to Cdk1 activity being greatly reduced (Irniger & Nasmyth, 1997). APC/C's activation at the onset of M phase leads to the ubiquitination Cyclin A during the prometaphase. Cyclin A is tasked with keeping Weel inactive. Weel is an inhibitor of CyclinB/Cdk1; and CyclinB/Cdk1 activity is bolstered as long as Weel remains inactive (Deibler and Kirschner, 2010). Cyclin A is also implicated in the nuclear envelope breakdown (NEB), though its involvement is not necessary (Gong et al., 2007).

APC/C's namesake activity occurs at the end of metaphase, where anaphase is ushered in by the targeting of Securin and Cyclin B by the APC/C^{Cdc20}. Securin is a Separase inhibitor, which is a protease that is responsible for the cleavage of kleisin (a subunit of cohesin) leading to the disassembly of cohesin and the separation of sister chromatids (Alfieri et al., 2017).

CyclinB/Cdk1 is another inhibitor of Separase. Cyclin B's destruction by the APC/C^{Cdc20} reduces the phosphorylation-based inhibition of Separase (Stemmann et al., 2001). Active Separase has another function in the form of disabling Cyclin B and by extension Cdk1 (Gorr et al., 2005). It is necessary for this transition to occur properly, as this is where the sister chromatids are segregated and divided evenly between the daughter cells. Faithful segregation of chromosomes is critical for the new cells' or new organisms' function and survival.

By the late anaphase, there is a considerable shortage of mitotic cyclins due to their degradation via APC/C^{Cdc20}. This, combined with the direct inhibition of Cdk1 by Separase culminates in the activation of APC/C^{Cdh1}; as not enough Cdks are present to prevent Cdh1 from interacting with APC/C. APC/C^{Cdh1} then mediates the degradation of a wider range of substrates, including Cdc20 (Prinz et al., 1998).

APC/C^{Cdh1} is necessary for mitotic exit and the maintenance of G1. It does so primarily by targeting Cyclins A and B; loss of Cdh1 in cells results in an accumulation of cyclins and unnecessary cellular divisions (Sigrist & Lehner, 1997). Moreover, APC/C^{Cdh1} is also able to indirectly induce an increase in the levels of p27 and p21. The levels of these Cdk inhibitors are usually kept in check by a SCF ubiquitin ligase called Skp2, and its accessory protein Cks1. By degrading Skp2 and Cks1, APC/C^{Cdh1} permits an accumulation of p27 and p21 (Bashir et al., 2004). Impairing Cdh1 leads to stabilized Skp2 and degradation of p27, which results in shortening of G1 (Nakayama et al., 2000).

Furthermore, APC/C^{Cdh1} also indirectly prunes Cyclin D1 levels; this cyclin is an activator needed for S phase entry (Penas et al., 2012). By preventing entry into the S-phase APC/C^{Cdh1} can prolong the G1 phase. Overall, the APC/C^{Cdh1} functions to induce mitotic exit, in addition to maintaining G1, by allowing a significant decrease in Cdk1 activity during mitotic exit. APC/C^{Cdh1} subsequently remains active late in mitosis and throughout G1, with lower levels in late G1 and S-phase, where its main role is relegated to the maintenance of low levels of mitotic Cdk activity. This enables the resetting of replication origins for the next rounds of DNA replication in the S phase (Pesin & Orr-Weaver, 2008; Penas et al., 2012).

The two APC/C activators have an opposing activity profile. APC/C is switched on in early stages of mitosis by Cdc20, where the APC/C is phosphorylated and Cdh1 activity is low. With Cyclins themselves being targets, APC/C^{Cdc20} activity leads to a reduction of Cyclin B, which in turn upregulates Cdh1. Increased levels of Cdh1 give rise to APC/C^{Cdh1} activity, which in turn allows the ubiquitination of Cdc20, inducing inactivation of APC/C^{Cdc20}. According to Alfieri et al. (2017) this switching of co-activators, from Cdc20 to Cdh1, serves two main functions. Firstly, by having similar overlapping but distinct substrate specificities, APC/C^{Cdc20} and APC/C^{Cdh1} can destroy specific cell cycle regulators during the different phases of their activity, thus permitting an ordered progression through the cell cycle. Second, Cdc20 and Cdh1

can be individually controlled by different regulatory mechanisms, making the system more flexible and robust. (Alfieri et al., 2017).

APC/C is an incredibly crucial piece of cellular machinery, but what controls the great controller? Three main mechanisms exercise control over the APC/C: the control of activator protein levels, APC/C inhibitory proteins, and phosphorylation. Phosphorylation of Apc1 subunit dissuade Cdc20 from associating with the APC/C; this phospho-regulation is performed by Cdk1 or Plk1 (Kraft et al., 2003). Alternatively, Cdh1 itself can be prevented from interacting with the APC/C by its Cdk-mediated phosphorylation (Zachariae et al., 1998).

Rca1 (in *Drosophila*) and EmiI (in vertebrates) are inhibitors of APC/C^{Cdh1} and therefore function to stabilise Cyclin A in the S phase (Dong et al., 1997; Reimann et al., 2001). *In vitro*, Emi1 can suppress activities of both APC/C^{Cdc20} and APC/C^{Cdh1}, but its main purpose is most probably to inhibit APC/C^{Cdh1} during the S and G1 phases preventing re-replication (Machida & Dutta, 2007). A maternal paralogue of EmiI, the Emi2 (or ErpI) blocks APC/C in a similar fashion to EmiI. This maternal paralogue functions to arrest eggs awaiting fertilisation during the metaphase of meiosis II (Schmidt et al., 2005).

By monitoring unattached microtubules and kinetochores, the spindle assembly checkpoint (SAC) also plays a major hand in silencing the APC/C. Until all the kinetochores are securely attached to their appropriate spindle, the “wait anaphase” signal produced by the SAC does not dissipate. The mitotic checkpoint complex (MCC) is the SAC enforcer and a highly potent inhibitor of APC/C^{Cdc20}. It is a multi-protein complex (chief among them being the Mad and Bub family of proteins) that is assembled near the kinetochores, unattached kinetochores catalyse the formation of the MCC (Lara-Gonzalez et. al., 2012; Yamano, 2019).

Meiosis

Meiosis is a specialized form of cell division that only occurs in the germ line cells of sexually reproducing organisms and is tasked with the production of gametes (sperm and egg cells). A single parent cell will segregate in two separate division events into four haploid daughter cells (the first division separates the homologous chromosomes which is immediately

followed by a second division involving segregation of sister chromatids without an intervening S-phase). Each daughter cell gets a maternal or a paternal copy of each chromosome. This differs from mitosis which culminates in two genetically identical diploid daughter cells. More importantly, meiosis sets up conditions for crossing over to take place. This is the exchange of genetic material between homologous chromosomes which occurs at the chiasmata. Meiosis further digresses from mitosis by producing gametes whose union at a fertilization event produces a genetically unique cell.

Strict dominion over meiosis is important for proper production of viable gametes. By nature, the meiotic divisions that constitute oogenesis (in multicellular organisms) need to be coordinated with growth and development of the oocyte, allowing for oocyte differentiation, and ensuring that completion of meiosis coincides with the fertilization event. To this end, the oocyte arrests during two separate phases of meiosis. The first time is in prophase I where release from this suspension happens through oocyte maturation. The second arrest happens during metaphase I or metaphase II (depending on the species); the oocyte is only released at an oocyte activation event. In *Drosophila*, the early mitotic divisions that occur after fertilization are completed without cytokinesis; therefore, come fertilization the oocyte must quickly inactivate meiotic regulators in order to prevent them from obstructing embryogenesis.

APC/C in meiosis

Both meiosis and mitosis use the same cellular machinery and operations to achieve drastically different results. Meiosis can be thought of as a modified version of mitosis tasked with producing twice the number of cells, each with half the number of chromosomes. This, at the very least, necessitates the activation of the APC/C twice for the separation of the sister chromatids.

To produce four haploid daughter cells, in meiosis the parent cell must undergo a second round of chromosome segregation without a S phase in between. To further complicate things, during the first meiotic division (meiosis I) pairs of homologous chromosomes must be separated (as opposed to pair of sister chromatids). The source of tension in this part of the process comes

from the chiasmata and cohesion between the arms of the chromosomes where crossing over is occurring. Only in meiosis II sister chromatids are pulled apart at the centromere (similar to mitosis). Two detachment events of meiosis I and II require Separase to be activated twice, each time through APC/C mediated Securin destruction (Pesin & Orr-Weaver, 2008).

Telophase and cytokinesis quickly mark the end of mitosis/meiosis, which is characterized by uncoiling of the chromosomes (which makes them diffuse and less compact), reformation of the nuclear envelope and breakdown of the spindle assembly. The absence of interphase in between the two meiotic divisions calls for specialized control over the APC/C activity. Enough Cyclin B-Cdk1 activity must be allowed for these processes of meiosis to occur smoothly, all while preventing DNA replication (Pesin & Orr-Weaver, 2008).

Given the monumental implications of meiotic divisions, the regulation of APC/C is critical. The APC/C must only be activated at certain points during meiosis, whereas it needs to remain dormant between the two meiotic divisions to allow for cyclins and Securin accumulation. This is of particular importance during oogenesis, where the cell enters a meiotic arrest twice. Once in prophase I, where it arrests to allow for crossing over to occur. Unsolicited APC/C activity here might cause loss of proper chromosome cohesion. The second arrest is at metaphase II where inhibition of the APC/C by EmiII prevents premature onset of anaphase (Pesin & Orr-Weaver, 2008).

Meiosis-specific APC/C coactivators have expanded our knowledge of the cell cycle regulation during oocyte development. In *Drosophila*, both female and male meiosis-specific APC/C activators have been uncovered. *Cort* mutants cannot finish meiosis and arrest at metaphase II (Lieberfarb et al., 1996; Swan & Schüpbach, 2007). On the other hand, *Fzy* mutants arrest later, in anaphase II, where Fzy is required for the destruction of Cyclin B at the meiotic spindle (Swan & Schüpbach, 2007). In contrast. Jacobs et al. (2002) have found what has been dubbed Fzr2, which is a Cdc20/Cdh1 homolog expressed specifically in male meiosis. It is able to activate the APC/C, as the misexpression of Fzr2 rescues a mutation in *fzr/cdh1*, and causes degradation of Cyclin B (Jacobs et al., 2002).

Cort

Cortex (Cort) is a female *Drosophila* meiosis-specific APC/C co-activator. *Cort* encodes a distant relative of the Cdc20/Cdh1 family of proteins. *Cort* expression is confined to a narrow time window during development. *Cort* mRNAs are highly expressed in the ovaries and are subsequently transferred into the egg along with the other maternally deposited mRNAs and proteins. *Cort* mRNA is unmasked (along with other maternally deposited mRNAs) in a cytoplasmic polyadenylation event that transpires at oocyte activation in stage 14 of oogenesis (Pesin & Orr-Weaver, 2007).

Cort mRNA is present up to 1 hour into embryogenesis, after which its levels drop dramatically to the point where *cort* transcripts are undetectable. Egg activation prompts the destabilization of maternal mRNAs, and *cort* mRNA is no exception, as it is deadenylated in a Ccr4-dependent manner, the Cort protein is also rapidly destroyed during this time. *Cort* expression is absent in males (Pesin & Orr-Weaver, 2007).

An N-terminal C-box and a C-terminal IR tail connect Cort to the APC/C; this physical association leads to the activation of APC/C. A meiosis-specific APC/C activator (i.e., Cort) potentially offers a way through which meiosis can be regulated, without having any spillover effects on embryogenesis/mitosis (Pesin & Orr-Weaver, 2007).

As it is a female specific activator of APC/C, Cort plays an essential role in meiosis progression and egg activation. Since APC/C is compulsory for the degradation of cyclins and Securin, females *cort* mutant are viable, but sterile, producing eggs that are arrested in meiosis II. Furthermore, they display increased levels of Cyclin A, B and B3 (Swan & Schüpbach, 2007). Cort also has roles outside of cell cycle, which are just as important as its task within the cell cycle. These include roles in translation; in *cort* mutants polyadenylation and translation of *bicoid* and *toll* is hampered (Lieberfarb et al., 1996). These two are part of the early embryonic patterning genes. Moreover, a great deal of early embryonic development hinges on the mRNA deposited into the egg by the mother. At the midblastula stage, where the zygotic genes start to become active and take over, those maternal mRNAs are destabilized. *Cort* mutants fail to undergo this destabilization, meaning *cort* has implications in maternal transcript destabilization. Finally, in *cort* mutants, long microtubules usually seen in the cortex of the egg, which are

typically depolymerized after egg activation, fail to be cleared out. This implies that Cort has a role in removing the cortical microtubules from the cytoplasm after egg activation (Page and Orr-Weaver, 1996).

Cort and Cdc20/Fzy play redundant and non-redundant roles in female *Drosophila* meiosis. Mutations in both of these cause arrest at different points of meiosis II; *cort* mutants arrest earlier at metaphase II whereas *fzy* mutants arrest at anaphase II. However, *cort* and *fzy* double mutants arrest much earlier, in meiosis I. This genetic redundancy is also manifested in the preference of Cort and Fzy for different substrates. Wherein APC/C^{Cort} can target both Cyclin A and B, APC/C^{Fzy} only has a predisposition towards Cyclin B. The spatiotemporal preference for Cyclin B also differs. Fzy is needed for degradation of Cyclin B associated with the spindle microtubules, whereas Cort is responsible for spindle midzone degradation of Cyclin B (Swan & Schüpbach, 2007).

The transition from oocyte to embryo is a critical process in development, even though its complete understanding still eludes us. The oocyte must switch from meiosis to mitosis once it starts to transition into the embryo. This switch is supported by various proteins and structures that were produced during gametogenesis and are bestowed to the embryo by both the egg and the sperm. For instance, the centrosome needed for spindle formation is brought in by the sperm; whereas the stockpile of nutrients, mRNA and the translation machinery that power the quick divisions of the zygote are provided by the egg. Proteins exist that are exclusively used during meiosis; overexpression or misexpression of these proteins leads to mitotic cell cycle deficits. Thus, a removal of these meiotic proteins is required for oocyte-to-embryo transition (Whitefield et al., 2013). One such protein is Matrimony (Mtrm). APC/C^{Cort} targets Mtrm for destruction at the oocyte to embryo transition. If excess Mtrm is not removed from the early embryo it leads to various developmental defects. Mtrm itself is an inhibitor of Polo kinase, which is another important regulator of mitosis and meiosis, and has been implicated in proper chromosome segregation, centrosome dynamics and cytokinesis. With such diverse roles in the cell cycle, up-regulation of Polo kinase is a hallmark of many human cancers (Whitefield et al., 2013).

Sex determination in *Drosophila*

A ubiquitous feature in eukaryotes is the determination of sexual morphology. The male or female ruling is done based on genes that reside on the sex chromosomes. The human sex determination system is the XX/XY system, which can also be found in most other mammals. In this system, females have two copies of the X chromosome whereas males have an X chromosome and a Y chromosome. The *sry* gene residing on the Y chromosome initiates the development of male physiology and thus is the determinant of "maleness" in humans (Larney et al., 2014).

Drosophila sex determination follows a version of the XX/XY system, where the males are XY, containing a single copy of the X chromosome; and females are XX. The Y chromosome has nothing to do with sex determination and is shorter with much fewer genes. Sex is determined by an X chromosome counting system which results in the transcription of the *Sex lethal (Sxl)* gene via X-linked signal elements (XSE). These XSEs are directly transcribed from the X chromosomes, meaning that their concentration is directly proportional to the number of X chromosomes the embryo has. Under normal conditions, a maternally deposited repressor maintains inhibition over the transcription of *Sxl*. This repressor called Groucho (Gro) together with its corepressor Deadpan (Dpn), establish a threshold of inhibition over *Sxl* (Salz & Erickson, 2010; Verhulst et al., 2010).

Sxl is said to be the female switch; in an XX individual *Sxl* in ON, as the requisite amount of XSEs needed to transcribe a sufficient quantity of Sxl can only be achieved in an individual with two X chromosomes. Sxl is a splicing factor and associates with general splicing factors U2AF and Sans-fills (SnF), among others, to regulate splicing of target RNAs, including its own. *Sxl* is also one of the earliest genes that is transcribed in the zygote and its expression oversees all aspects of female development, all while simultaneously preventing male-specific dosage compensation. Conversely in a XY individual, *Sxl* is OFF, and a male-specific dosage compensation complex is activated that upregulates the transcription of the singular male X chromosome two-fold (Figure 1.3). This epigenetic tuning of the X chromosome is in contrast to humans, where in females, one of the two chromosome is deactivated; the X chromosome that is deactivated is chosen completely at random (Salz & Erickson, 2010).

Sxl is transcribed from two promoters, the first one of which is an early transient promoter dubbed the *SxlPe*. Activation of this early promoter is a direct transcriptional response to the X chromosome dose (via XSEs). The four proteins that constitute the XSEs are: Sisterless A (SisA), Scute (SisterlessB or SisB), Unpaired and Runt. SisB and Unpaired bind to their maternally deposited heterodimeric partner Daughterless (Da) and directly activate the *SxlPe*. SisA and Runt also bind to and activate *SxlPe*, but their heterodimeric partners are unknown (Salz & Erickson, 2010).

The "early" *Sxl* promoter (*SxlPe*) threshold is first achieved during cycle 12 in the blastoderm and is maintained for around 40 minutes until it shuts off in cycle 14. The *SxlPe* provides the developing female with an early burst of the *Sxl* mRNA and Sxl protein. The XSEs together must overcome the repression threshold established by Gro and Dpn. An XX individual can produce and accumulate enough XSEs by cycle 12 that they can now effectively interfere with the Gro-mediated *SxlPe* repression, and eventually activate it. This cannot happen in XY individuals as they are unable to transcribe enough XSEs in time. Once *SxlPe* is activated, a switch to a maintenance promoter is organized, meanwhile XSEs continue to oppose the Gro-mediate repression and continue to stimulate high levels of *Sxl* transcription from the *SxlPe*. Even though in XY individuals the XSE levels also keep rising throughout cycle 13 and 14, *SxlPe* remains silent as Gro repression is reinforced by the zygotic Dpn repression. Dpn repression adds a layer of protection in this system, ensuring that it is "leak" proof. (Salz & Erickson, 2010).

As hinted earlier, the second major target regulated by the XSEs is the maintenance promoter (*SxlPm*). In contrast to the *SxlPe*, the *SxlPm* gets turned on at gastrulation and lasts all through adulthood; it is also expressed in both males and females. SisB and Runt, in conjunction with the maternal Da protein, are mainly responsible for acting on the enhancer to this promoter and its activation. The maintenance promotor permits the *Sxl* to convert the transient X-chromosome dose signal into a more permanent irreversible signal, that can regulate its own expression at the level of splicing. Without Sxl protein (as is the case in males) the transcripts produced from the *SxlPm* contain a translation terminating stop codon in the third exon which makes them impotent. On the other hand, the presence of the Sxl protein (generated from the *SxlPe*) allows for this third exon to be ignored, in a process known as exon skipping. The *Sxl* pre-

mRNA has Sxl binding sites (poly-U sequences) around 200 nucleotides upstream and downstream of this third exon. Spliceosome assembly starts on the 5` site where the U1 of the snRNPs are built; meanwhile the 3` site is where the U2AF is set up. Overall, Sxl blocks splicing by interacting with general splicing factors bound to their authentic splice sites (Salz & Erickson, 2010).

This overlap between *SxlPe* and *SxlPm* exists to allow sufficient quantities of Sxl and its pre-mRNA to be present together to efficiently engage splicing control early on in the blastoderm stage. While at the same time providing a sufficient pulse of Sxl that can trigger the positive feedback loop for the productive splicing of *SxlPm* transcripts, this feedback loop then maintains the female *Sxl* ON status thereafter, throughout adulthood. If that initial burst of Sxl from *SxlPe* is not present, then this positive feedback loop cannot be engaged and the *SxlPm* continues to produce the non-functional *Sxl* transcripts (Salz & Erickson, 2010).

Sxl acts on the pre-mRNA of the downstream *transformer* (*tra*) gene to promote its productive splicing and bringing about a functional Tra protein (Tra^{f}) (Figure 1.3). Sxl binds to a polypyrimidine tract on the first intron of the *tra* pre-mRNA and directs the general splicing factor U2AF to use the female-specific 3` splice site in exon 3 instead of the non-sex-specific 3` splice site in exon 2. This splicing of *tra* transcript yields a functional Tra protein. Since Sxl is only active in females, a functional Tra protein is only present in females, whereas males have a truncated non-functional Tra protein (Tra^{m}). Wildtype females possess both male and female isoform of the Tra protein, in roughly equal amounts, as only about half of the *tra* pre-mRNA are processed by Sxl (Verhulst et al., 2010).

The Tra protein is a splicing factor that oversees the two downstream arms of morphology and behavior. Tra along with its partner Tra2 form and activate a complex that is responsible for the splicing of *doublesex (dsx)* in the morphology arm of sex determination (Figure 1.3). Tra splicing of *dsx* leads to the formation of the female specific Dsx^{F}; which is a transcription factor that acts on multiple downstream genes that result in female sexual characteristics and behavior, along with the suppression of male sexual characteristics, ultimately controlling the female somatic sexual differentiation (Burtis & Baker, 1989). Default splicing of *dsx* (without Tra) leads to male specific Dsx^{M}, which activates male specific sexual characteristics while supressing female characteristics (Pomiankowski et al., 2004).

Similarly, Tra and Tra2 directly regulate *fruitless (fru)* splicing, in the behavior arm of sex determination (Figure 1.3). Tra splicing of *fru* leads to a non-functional protein; Fru is important in determining male courtship behavior and sexual orientation. Female *tra* mutants display male-specific courting behavior and splicing of *fru* (Heinrichs et al., 1998).

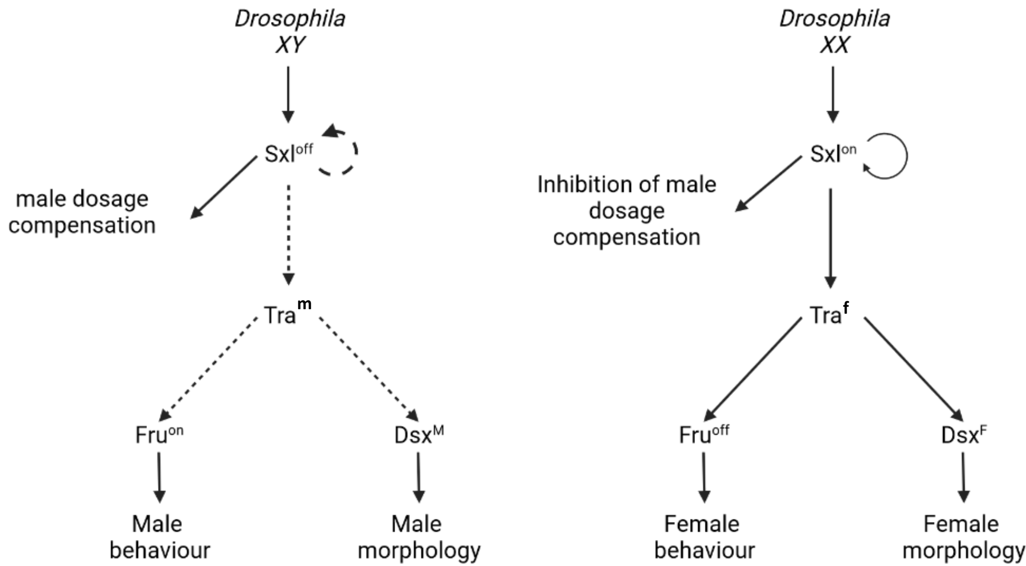

Figure 1.3. *Drosophila* sex determination pathway; individuals with 2 X chromosomes have expression of splicing factor Sxl. Sxl acts on the pre-mRNA of downstream *tra* and itself, and at the same time inhibits male dosage compensation. Dotted lines indicate pathways that are not on.

In addition to being the master sex switch, Sxl is also involved in dosage compensation. A male-specific-lethal-2 (Msl-2) can only be fabricated in males, while in females this male-specific dosage compensation complex is left unassembled. The Sxl protein represses the production of the Msl-2 protein. This is accomplished in two ways; in the nucleus where Sxl is involved in intron retention and in the cytoplasm where it inhibits translation (Salz & Erickson, 2010).

Intron retention works by having 5` and 3` intronic Sxl binding sites that are in close proximity to splice sites. Sxl binding to these sites hinders intron recognition via the displacement of U2AP at the 3` site, and the displacement of U1 snRNP at the 5`splice site. Intron retention ensures that there are Sxl-binding sites in the mature *msl-2* mRNA. These sites

later allow Sxl to repress *msl-2* translation. Mutant variants that retain the introns while blocking splicing cannot impede *msl-2* function or regulation (Salz & Erickson, 2010).

Sxl interferes in translation in two ways by the virtue of the two Sxl-binding sites. Bound to the 3` site, Sxl blocks the recruitment of 43S ribosome to the 5` site of *msl-2*. Whereas, Sxl binding to the 5` site restricts the 43S complex from scanning the sequence and from reaching the AUG start codon (Salz & Erickson, 2010).

The active form of Msl-2 functions by enhancing the transcriptional activation of the lone X-chromosome of the male cells. Working with other partners, it forms the dosage compensation complex (DCC). The DCC binds to various sites on the male chromosome, altering the chromatin structure to permit heightened activation (Kelley et al., 1995). Turner (1992) showed that it is likely that H4 histone molecules are acetylated at particular sites that allow for higher transcriptional activation of the male X chromosome (Turner, 1992).

***Cort* misexpression and its effects on female sexual development**

Investigations into the misexpression of *cort* were done by Osama Batiha, a former PhD student from our lab. He used the UAS/GAL4 system to drive expression of transgenic *cort*. In this system a transcription factor (Gal4) acts on the upstream activation sequence (*UAS*) to drive expression of a gene downstream of the *UAS*. The Gal4 transcription factor is regularly fused to enhancer elements that permit expression at particular times (Duffy, 2002). For example, in *Drosophila, engrailed* is necessary for the development of posterior compartment of wings. A Gal4 fused with *engrailed* regulatory sequence will only be expressed in the wings along-side *engrailed*. A transgene contains *UAS* and our gene of interest. The gene itself is fused to an epitope tag that enables its detection.

Batiha (2013) discovered that misexpression of *cort* (using a ubiquitously expressed *da-Gal4*) has a rather peculiar effect on the adult *Drosophila*. Whereas the misexpression of *fzy* or *fzr* is lethal, *cort* misexpression is not; instead, it has an effect on the sexual morphology of adult flies, transforming genetically female (XX) flies to ‘male-like’ flies. It should be noted that *cort* expression is normally limited in the female germline, however its expression throughout

development (i.e., outside the usual time window of its expression) leads to this particular transformation (Batiha, 2013).

Sexual dimorphism in *Drosophila* manifests itself in various ways: females are generally larger in size than males. They have partial pigmentation in the posterior abdominal segments which prevent darkening (Figure 1.4 A and B). Finally, they lack sex combs, which are typical male physical characteristics present on middle of each of the two forelegs (Figure 1.4 A" and B"). Male adult flies misexpressing *cort* are comparable to wildtype males. However, females flies that are misexpressing *cort* (*HA-cort/da-Gal4*) are transformed to male-like individuals. These individuals have genitalia closer in appearance to male genitalia (Figure 1.4 C') and have pigmentation of the lower abdomen (Figure 1.4 C, arrows). They also possess partial sex combs, the bristles of which tend to be less organized (Figure 1.4 C"). The aspect of female morphology that is left mostly unchanged is the size, as these transformed females tend to be larger than males (Figure 1.4 compare A and C). The same phenotype was seen when *cort* was misexpressed using a different Gal4 driver (act-Gal4), or even a different line of *HA-cort* (Batiha, 2013).

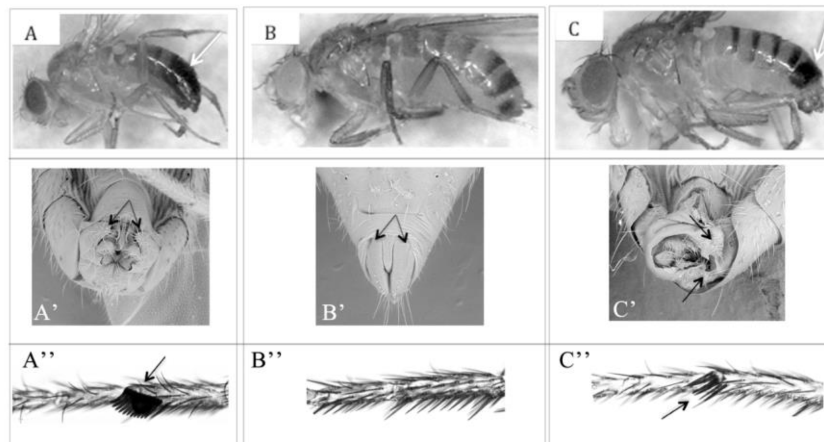

Figure 1.4. Misexpression of *cort* results in genetically female individual transforming to male-like individuals. **(A)** A male *Drosophila*, arrow pointing towards the dark pigmentation on the posterior abdomen segment. **(A')** external genitalia in males showing distinct male parts like the male claspers marked by arrows. **(A'')** Sex combs in males, present on each foreleg. **(B)** A typical *Drosophila* female lacks abdominal pigments and is larger in size. **(B')** typical female genitalia, arrows point out the vaginal bristles. **(B")** Females do not have sex combs on their forelegs. **(C)** *cort* misexpression in female *Drosophila*; dark pigmentation and similar in size to females; **(C')** absence of female genitalia and presence of male claspers (arrows); **(C")** partial sex combs. (Batiha, 2013)

In *Drosophila,* sexual identity is set up in a cell autonomous fashion. Thus, a transformed individual can either display mosaicism (i.e., both male and female cells are present together) or be a true intersex (partial transformation of each individual cell). One way to discern between these two possibilities is to study the phenotype of the single cells that produce the individual bristles on the sex comb. Around 10 specialized cells constitute a sex comb, each cell spawns a specialized thickened bristle on the forelegs of the males. A further distinction of these sex combs cells is that they are rotated relative to other bristles on the leg. Since each bristle sprouts from a single cell, the number of bristles in the sex comb would indicate a degree of mosaicism, whereas the reduced thickness would imply intersex. Examination of the sex combs revealed the bristles to be lower in numbers with reduced thickness (Figure 1.4 compare A'' to C''), meaning that the transformed individuals are both mosaic and true intersex (Batiha, 2013).

Which part of the sex determination pathway does *cort* affect?

Cort exists outside of the sex determination pathway of *Drosophila,* so how is it able to completely change the sexual fate of an individual? By looking into how Cort effects the splicing cascade in the sex determination pathway, we can establish how *cort* misexpression leads to the female to male transformation. Sxl is the master regulator of sex determination in *Drosophila* and is one of the earliest genes to be activated, so the effect of *cort* misexpression on Sxl should be examined first. Batiha (2013) used primers that could discern between male and female isoforms of *Sxl* and found that female *HA-cort* only had the female isoform of *Sxl*. This means Cort cannot be interfering with Sxl function (Batiha, 2013).

Furthermore, Cort could not be targeting Sxl protein or its activity since maintenance of *Sxl* female splicing depends on the activity of Sxl protein itself. Failure to downregulate X-chromosome gene expression as part of the dosage compensation means that absence of Sxl is lethal to females. With *HA-cort* flies being viable, this further validates that Cort induces a sex transformation elsewhere in the sex determination pathway. The body size of flies is also under Sxl control, and the size of the transformed female flies looks very similar to wildtype females.

This further proves that Cort could not be acting at or above Sxl in the sex determination pathway (Batiha, 2013).

When looking at Tra and *tra* splicing, the gene immediately downstream of *Sxl*, similar results were observed. *HA-cort* females gave results very similar to wildtype females, i.e., both isoforms of *tra* were observed. Females having both male and female isoforms of *tra* is possibly because Sxl cannot process all the transcribed pre-mRNAs. Meanwhile, in males, only the male isoform is made. The same conclusion can be drawn here as well, *cort* misexpression probably effects the sex determination pathway on a level lower than that of *tra* splicing. Unfortunately, it is difficult to be confident in this result, as the presence of male isoform in *HA-cort* females could be a result of leakiness of the female specific splicing or could also reflect a partial transformation (Batiha, 2013).

Examination of *dsx* and *fru*, the two targets downstream of Tra, showed more straightforward results. In each case the *HA-cort* females showed isoforms from both males and females. Wildtype flies never show both isoforms, it can be either male or female isoforms. Therefore, it was concluded that misexpression of *cort* causes interference somewhere above *dsx* and *fru* splicing, and somewhere below *sxl* splicing (Batiha, 2013).

Failure of the *tra* splicing analysis to show conclusive results prompted another look into misexpression of *cort* at the level of *tra*. A genetic epistasis approach revealed that male flies coexpressing tra^f and *cort* appear to show the transformation phenotype and are in-between or intersex. On the other hand, the female flies that are coexpressing tra^f and *cort* appear as normal females. This means that *cort* is at least partially epistatic to *tra. Cort* epistasis implies that Cort acts downstream in the *Drosophila* sex determination pathway and that Cort might be involved in the transcriptional repressing of Tra. Partial epistasis can be expected if transgenic Cort is unable to completely repress Tra (Batiha, 2013).

Cort inhibits Tra as part of the APC/C

Cort is a co-activator of the APC/C, and as an APC/C co-activator its job is to identify and single out various substrates. In order to characterize if Cort causes the sex transformation

phenotype due to its interactions with the APC/C, Batiha (2013) generated a HA-tagged version of Cort that lacked the IR motif. The two amino acids of the IR motif are implicated in the interaction between APC/C and its other co-activators (Cdc20 and Cdh1) with the APC/C subunit Cdc27. *HA-cort*^{IR}*/da-Gal4* flies were unable to produce a significant sex transformation phenotype. Thus, it can be said that Cort's ability to induce sex transformation is likely due to its interaction with the APC/C (Batiha, 2013).

An additional test involved knocking down of a specific APC/C subunit. RNAi against the subunit Cdc23 were generated by Batiha (2013) and when *HA-cort* was coexpressed with *cdc23*^{RNAi} the resulting females appeared identical to wildtype females. This points towards the idea that the APC/C component of Cdc23 is required for the sex transformation activity of Cort (Batiha, 2013)**.**

Maternal Cort is important for regulation of zygotic sex determination

Siera and Cline (2008) found evidence for the ability of Tra to weakly initiate the *Sxl* positive feedback loop that is established in early blastoderm stage from the *SxlPm*. There is evidence that Tra directly promotes productive *Sxl* splicing, producing a functional Sxl protein, which then acts on its own mRNA to initiate the *Sxl* positive feedback loop (Siera & Cline, 2008). This provides us with an opportunity to put the APC/C^{Cort} mediated destruction of Tra into some context. Batiha (2013) formulated the idea that Cort has a biological role in the regulation of maternal Tra activity to prevent premature activation of *Sxl*, hypothesizing that this premature activation of *Sxl* could have deleterious effects on male development. To this end, Batiha (2013) investigated what a loss of maternal cort could do. He found that *cort* mutant flies produced male offspring that were atypical, with about 1% of them having abnormal or missing genitalia. In order to increase the penetrance of this phenotype, the genetic background was sensitized by crossing female *cort* mutants flies with males that had an extra copy of the *Sxl* gene (*Dp(1,Y)Sxl*$^{+}$). This sensitized genetic background was able to enhance the phenotype, with now 10% of the male progeny being abnormal. These male progeny displayed both male and female *Sxl* isoforms (Figure 1.5 G lanes 4 and 6), compared to control male, that only had the male

isoform of *Sxl* (Figure 1.5 G lanes 3 and 5). Possibly because *Sxl* expression is lethal in males, there was also a notable reduction in male viability, by almost 30% (Batiha, 2013).

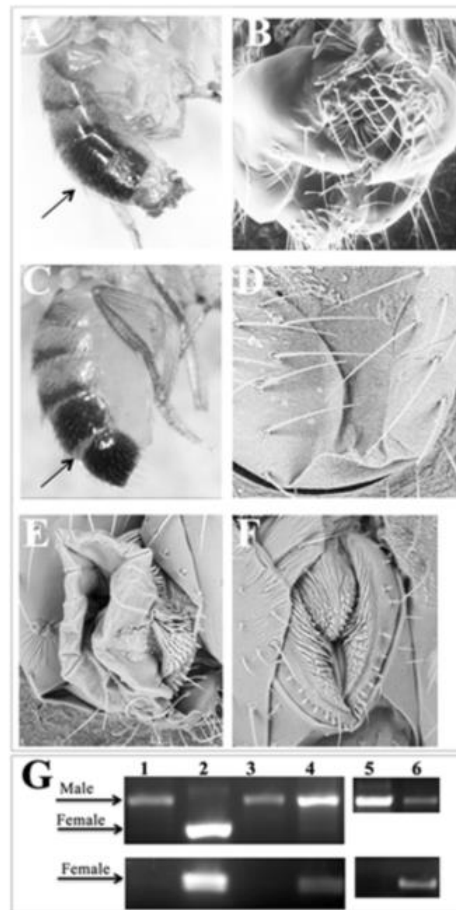

Figure 1.5. Maternal role of Cort, removal of maternal Cort leads to abnormalities in male progeny. **(A and B)** wildtype male featuring normal pigmentation (arrow) and normal male genitalia. **(C-F)** male progeny from *cort*^{QW}*/cort*^{RH}*;nanos-Gal4/HA-cort*^{IR} crossed to *Dp(1,Y)Sxl*$^{+}$ (a *Sxl* duplication) showing pigmentation with missing genitalia (arrow). **(D)** or highly abnormal genitalia **(E)**, or in very rare cases, abnormal looking female genitalia **(F)**. **(G)** RT-PCR analysis using *Sxl* primers. **(Lane 1 and 2)** wildtype male and female respectively. **(Lane 4 and 6)** two different samples run on different gels of *cort*^{QW}*/cort*^{RH}*;nanos-Gal4/HA-cort*^{IR} (in *Sxl* sensitized background) male progeny, showing both male and female isoform of *Sxl*. (Batiha, 2013)

Model for the role of maternal Cort and current hypothesis

The results described above lead Batiha to a model in which APC/C^{Cort} is tasked with the targeting of Tra for destruction. When extrapolating this finding to the germline we see a scenario where APC/C^{Cort} is tasked with strictly controlling maternal Tra activity, to make sure that it cannot establish the *Sxl* positive feedback loop. This could not be an issue for female development but an untimely initiation of Sxl production in males would be highly detrimental to their viability and sex determination. In light of these facts and discoveries, a model was proposed: Cort allows for the ubiquitination of Tra marking it for destruction, and that Cort has a biological role in regulating maternal Tra activity to prevent premature activation of *Sxl* (Figure 1.6).

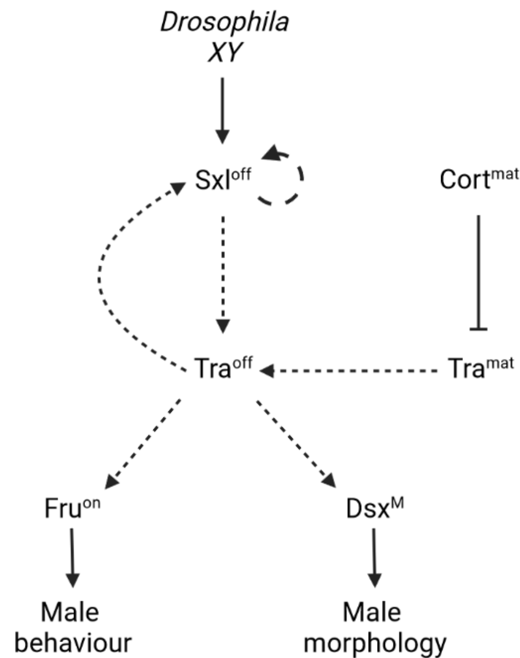

Figure 1.6. Our proposed model for the maternal role of Cort. In *Drosophila*, maternally expressed APC/C^{Cort} inhibits Tra activity. This safeguards against Tra-mediated splicing of *Sxl* transcripts in the early male embryo resulting in the initiation of the *Sxl* positive feedback loop which has disastrous effects on male viability and morphology.

The purpose of the present study was to build on the foundations set by Batiha (2013) and to explore the proposed model. My overall goal is to verify the discoveries previously made by Batiha, with my first objective being a look into the internal structures of transformed females to check for the effects of transformation. My second objective will be to show, through epistasis experiments, that *cort* has genetic influences upstream of *tra*. I will be employing the use of a different *tra* transgene and use the sex comb phenotype as a means of quantifying these findings. My third objective will be to investigate the role of APC/C in Cort mediated sex transformation, again using a different *tra* transgene, new controls and most importantly I will be quantifying the findings, as previously, visual examination of external genitalia was used as a qualitative indication of sex transformation. For the second objective, I will be directly confirming that Cort targets Tra for destruction. Given how Cort only functions as an APC/C co-activator and that APC/C is a ubiquitin ligase, we predict that overexpression of Cort will lead to a reduction in Tra

levels. We tested this by generating a flag tagged Tra and determining its levels in presence or absence of transgenic Cort.

For my fourth objective, I will also be expanding on our understanding of the maternal role of Cort and the regulation of zygotic sex determination. I will explore and quantify the affects of loss of maternal Cort on male development and viability. By testing ability of the XSEs *sisA* and *sisB* duplications to enhance the maternal phenotype (similar to *Sxl* duplication), I intend to show that these effects are due to a disruption of male sex determination and dosage compensation. With the present study I hope to expand our working knowledge on the APC/C and examine the multifaceted role of APC/C and Cort.

CHAPTER II: RESULTS

Cort acts at the level of Tra

The sex transformation phenotype caused by misexpression of *cort* manifests itself as complete or partial transformation of external genitalia and sexual characteristics (Figure 1.4). However, it is not known how this transformation effects the internal structures of the individual. Upon examination of the internal structures, it was discovered that transformation leaves the internal structures of XX individuals looking are more like male testes. Figure 2.1 A and B shows the internal structures from two XX individuals that are misexpressing *HA-cort*. This expression of HA-Cort in these transgenic flies was driven by *da-Gal4* (enables ubiquitous expression of transgene). Figure 2.1 C and D are wildtype male testes and female ovaries respectively. The wildtype testes appear as a coiled-up tube, whereas a typical ovary appears as a bunch of bananas.

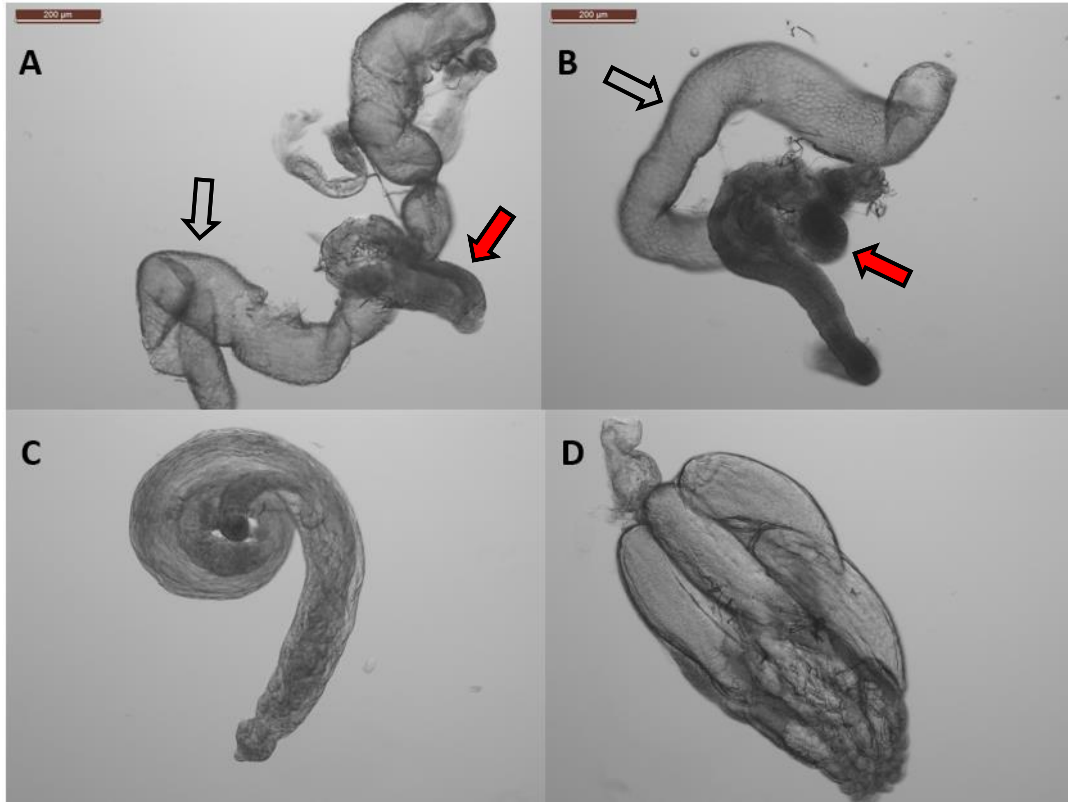

Figure 2.1. The internal structures of *cort* misexpressing and wildtype adults. **(A and B)** internal structures from two different transformed *XX; da-Gal4/UASp-HA-cort*^{wt}*(3,7)* individuals. Filled arrows point towards the pseudo-testes, hollow arrows point out the seminal vesicles. Misexpression of *cort* leads to the transformation of the internal sexual organs. Compare these to **(C)** a wildtype male testis (seminal vesicle removed) and **(D)** a typical wildtype female ovary. The ovary and testes exist in pairs but only one from each pair is pictured.

Results of earlier epistasis experiment showed a partial epistatic relationship between *cort* and *tra*^{f} (Batiha, 2013). This experimental design was repeated, but this time using a UAS Tra transgene (*UASp-tra*^{f}*-GFP)*. More importantly the results will be quantitated by determining the sex comb scores for individuals. Furthermore, we also repeat the *cort-tra* epistasis using *HA-cort*^{IR} to further test the idea that this mutation makes Cort unable to promote the sex transformation.

Both males (XY) and female (XX) flies were examined to establish this epistatic relationship. If *cort* were to act upstream of *tra* splicing, then *tra*^{f} should be completely epistatic to *HA-cort*. For example, if Cort were to affect *tra* transcription, it would have no effect on

transgenic Tra^f, and thus Tra^f would be able to transform males to females and rescue transformation of Cort mediated females to males. On the other hand, if *cort* acted at the level of Tra protein or Tra activity we could expect *HA-cort* to be epistatic to *tra^f*, depending on expression levels. In other words, HA-Cort would be able to act on transgenic Tra^f to reverse the transformation of males to females. Partial epistasis would be expected if Cort is unable to fully block Tra or Tra^f activity.

To quantitate the epistatic relationship between transgenic *cort* and *tra^f*, we quantitated the sex combs. For each individual the quality and quantity of sex combs was determined (See materials and methods). Sex combs are a male sexual characteristic (Figure 2.2 A); therefore, wildtype males tend to have an average sex comb score of about 10. Females do not posses sex combs, thus have a sex comb score of 0 (Figure 2.3 *yw*).

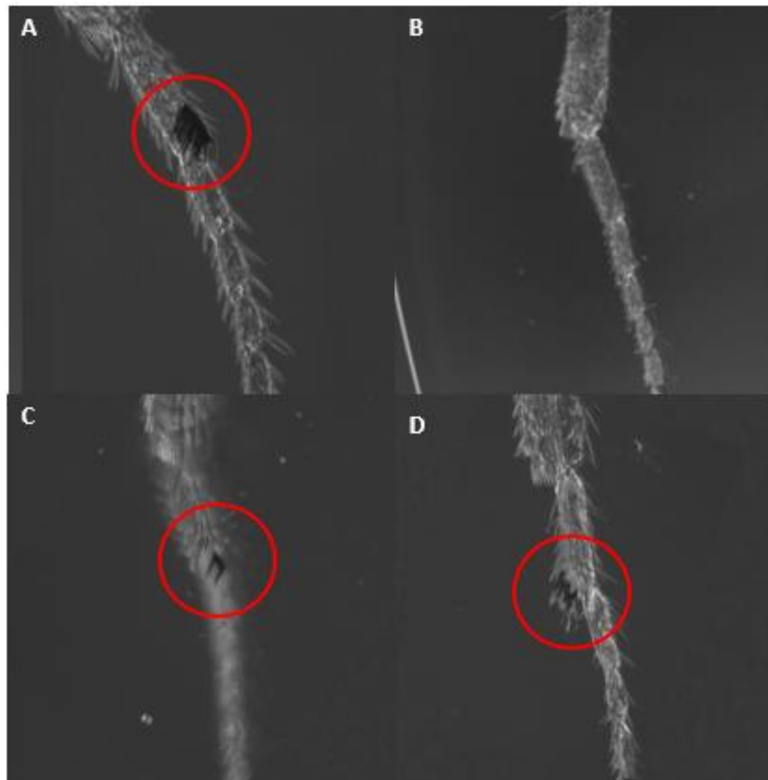

Figure 2.2. Sex combs on the forelegs of adult *Drosophila* (sex combs are highlighted). **(A)** a wildtype male. **(B)** a wildtype female, no sex combs present. **(C and D)** genetically XX; *da-Gal4/UASp-HA-cort^{wt}(3,7)* individuals displaying sex combs, various degrees of transformation are seen, with a sex comb score of 3 (C) and 6 (D) (see figure 2.3).

The expression of *HA-cort* and *UAS-tra-GFP* was driven by *da-Gal4*. The use of *UAS-tra-GFP* allowed us to ensure that the number UAS elements across conditions were balanced out, allowing us to be sure that effect on sex comb score is due to the different transgenes present *HA-cort* by itself caused the expected transformation phenotype, with females being transformed to males; this phenotype was defined by an increased sex comb score for genetically XX individuals (Figure 2.3 *GFP;HA-cort*). Examples of such transformation are shown in figure 2.2 (C and D), the sex combs are less organized and with some stubby bristles. This is likely attributed to the fact that somatic cells display both a mosaic and true sex transformation. Male sex comb scores, for the most part, are not affected (Figure 2.3 GFP;HA-cort M).

HA-cort^{IR} is a version of *cort* that has its IR tail motif deleted. As a result, its association with APC/C is expected to be greatly hindered; thus, Cort cannot carry out its function as part of the APC/C and has no capacity to cause the sex transformation (Batiha, 2013). Indeed, when *HA-cort^{IR}* and *tra^{f}* are coexpressed, the XY males are transformed to female-like individuals, displaying significantly lower sex comb scores (Figure 2.3 *tra-GFP;HA-cort^{IR}*). Going from an average score of 9.8 to 3.7. Females are not affected at all, as their sex comb scores are unaffected and remain 0.

When *HA-cort* and *tra^{f}* are coexpressed (Figure 2.3 *tra-GFP;HA-cort*), the sex combs scores for these flies are very similar to the sex comb score of flies that are expressing *HA-cort* alone (compare to figure 2.3 *GFP;HA-cort*). This is in huge contrast to when the flies are expressing *tra^{f}* and *HA-cort^{IR}* (compare to figure 2.3 *tra-GFP;HA-cort^{IR}*). These results imply that *cort* is at least partially epistatic to *tra*. Expression of either *tra^{f}* or *HA-cort* alone is associated with a unique phenotype, *tra^{f}* causes males to transform into females whereas *HA-cort* causes a female to male transformation. However, when the two are coexpressed, one of the phenotypes is more pronounced and masks the other, which our results indicate to be the transformation of females to males. This can only happen if *cort* expression affects the levels or the function of Tra protein (or acts further downstream of *tra* expression). Since it is a partial epistasis as *HA-cort* is not completely able to mask the *tra^{f}* phenotype, this means that HA-cort cannot be interrupting Tra transcription and in fact interferes in a post-translation manner.

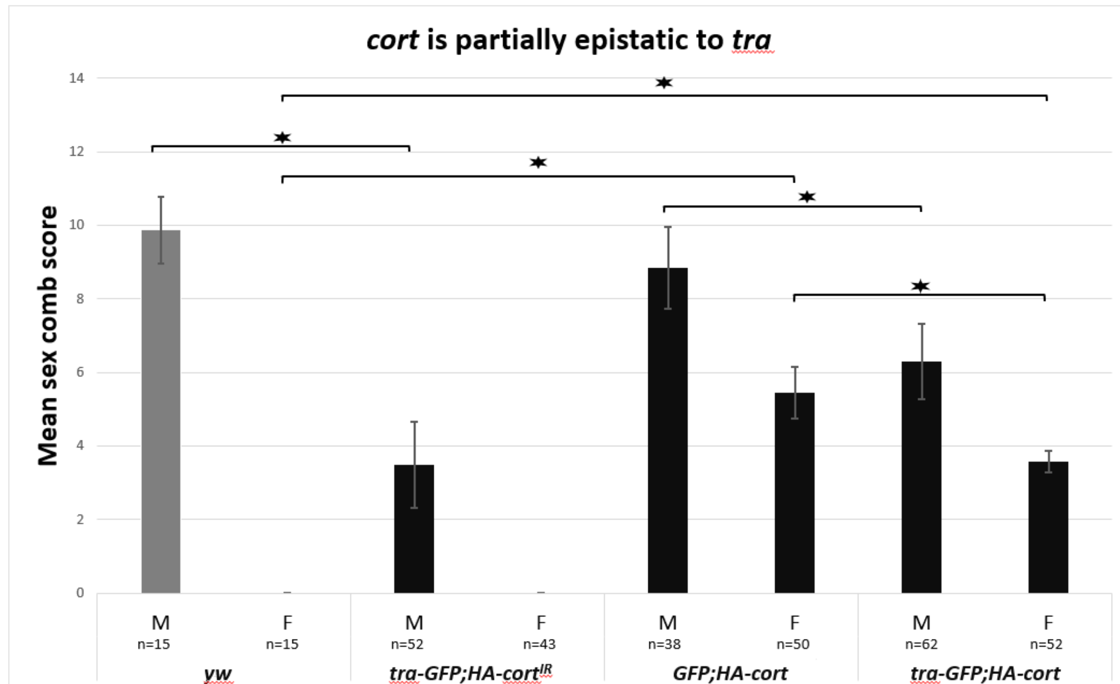

Figure 2.3. *Cort* is partially epistatic to *tra^{f}*. Characterization of phenotype done via the quantification of sex combs. Each individual hair bristle of the sex comb was counted and given a score, if the hair was thick and rounded at the end, it received a score of 1. If the hair was of partial length, it received a score of 0.5. For each individual fly, the sex comb score was the sum of all bristle scores. **(*yw*)** Wildtype males have sex comb score of about 10 whereas females have a sex score of 0. **(*tra-GFP;HA-cort^{IR}*)** coexpression of *UASp-tra^{f}-GFP* and *UASp-HA-Cort^{IR}* results in transformation of males to females, represented by the considerable lowering of mean sex comb score for males, females are unaffected. **(*GFP;HA-cort*)** expression of *UASp-GFP;UASp-HA-cort^{wt}(3,7)* alone, leads to the female to male sex transformation phenotype, characterized by females presenting a non 0 mean sex comb score. *UAS-GFP;UASp-HA-cort^{wt}(3,7)* stock was used in particular to ensure the UAS element was balanced out for all conditions. **(*tra-GFP;HA-cort*)** Coexpression of *UASp-HA-cort^{wt}(3,7)* and *UASp-tra^{f}-GFP*, sex comb scores for male and females are similar to *HA-cort* expression. In all cases, *UAS* transgenes were expressed under the control of *da-Gal4* ($* = p < 0.05$). A one-way ANOVA also showed that there was a statistically significant difference in the mean sex comb score between the groups ($F(5,12) = 38.3 \ \ p < 0.001$)

Sex transformation phenotype is because of APC/C^{Cort}

Cdc23 is an important part of the APC/C scaffolding module; it is implicated in the interaction with the IR tail motif present within the APC/C co-activators. Using RNAi to

knockdown *cdc23* should allow us to impair the function of APC/C. We predict that the ability of Cort to induce a sex transformation is likely due to its interactions with APC/C via Cdc23. *cdc23* RNAi should cause a loss of the sex transformation phenotype. Batiha (2013) arrived at the same conclusion but, however, he only corelated an absence of sex transformation with presence of $cort^{IR}$ by doing a qualitative analysis of external genitalia. I solidify and expand on this finding by corelating sex comb scores with $cdc23^{RNAi}$ and $HA\text{-}cort^{IR}$ coexpression. Quantification of sex combs offer a look into the degree of transformation, as *HA-cort* mediated sex transformation is seldom a complete transformation. It should further test our prediction that *HA-cort* mediated sex transformation is due to the actions of APC/C^{Cort}.

Since APC/C is crucial for healthy development of embryos, impairing the functions of APC/C with *cdc23* RNAi causes lethality in most larvae and pupae. This lethality can be circumvented by carrying out the experiment at lower temperatures. Indeed, at 18°C some male and female larvae escape this lethality and grow into healthy adults. Under these conditions we tested the requirement for Cdc23 for the Cort mediated sex transformation. We found that when $cdc23^{RNAi}$ is expressed by itself the adults appear similar to wildtype (Figure 2.4 compare *yw* to $cdc23^{RNAi}$). Alternatively, expression of *HA-cort* alone causes the sex transformation, with females now possessing sex combs; males are relatively not affected much by the presence of Cort alone (Figure 2.4 compare *yw* to *HA-cort*). Coexpression of *HA-cort* and $cdc23^{RNAi}$ results in females having a similar sex comb score to wildtype (Figure 2.4 $cdc23^{RNAi}$;*HA-cort*), with results being similar to when expressing $cdc23^{RNAi}$ alone. With APC/C not working as intended, Cort is unable to produce the sex transformation it typically does. We conclude that the sex transformation phenotype is likely due to Cort's association with APC/C.

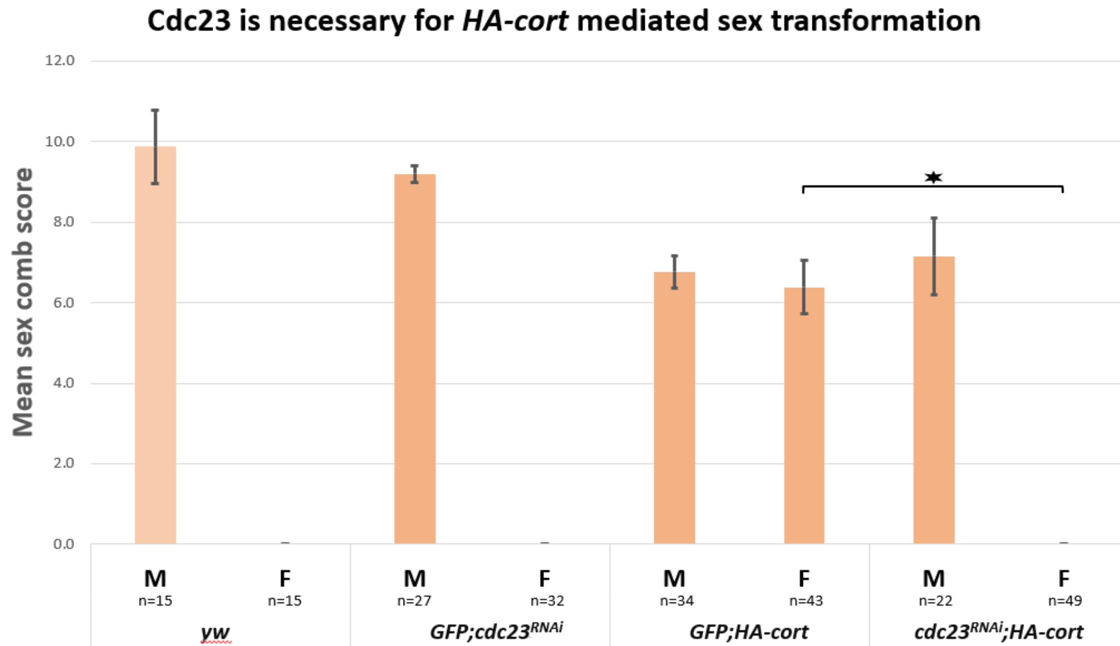

Figure 2.4. Impairing APC/C subunit Cdc23 with RNAi leads to loss of *HA-cort* induced sex transformation, as determined by the of sex comb score. (***yw***) Mean sex comb score for wildtype males and females. (***GFP;cdc23^{RNAi}***) *UASp-GFP*;*UASp-cdc23^{RNAi}* sex combs scores are similar to wildtype. (***GFP;HA-cort***) *UASp-GFP*;*UASp-HA-Cort^{wt}(3,7)* causes female to male sex transformation; females now have mean sex comb score of approximately 6.4. (***cdc23^{RNAi}:HA-cort***) coexpression of *UASp-cdc23^{RNAi}* and *UASp-HA-Cort^{wt}(3,7)* does not result in a sex transformation phenotype; females have means sex comb score of 0. Reduction in male sex comb score is likely a consequence of the removal of Cdc23. Expression of all *UAS* transgenes was under the control of *rn-Gal4* with crosses carried out at 18 °C (* = $p<0.05$). A one-way ANOVA also showed that there was a statistically significant difference in the mean sex comb score between the groups ($F(5,12) = 28.8, p < 0.001$).

With evidence indicating that *cort* genetically acts on *tra* in the Drosophila sex determination pathway, we moved on to test how *cort* expression effects Tra levels. Since Cort serves as a co-activator of the APC/C we predicted that in presence of Cort, Tra protein levels will decrease. Two different approaches were employed; the first approach involved an antibody against Tra^{f}. No specific antibody against the Tra^{f} protein exists, thus we generated our own. This novel polyclonal antibody was manufactured by Biomatik from the following peptide sequence that we provided: YHGRSSERDSRKKEH-Cys. The effectiveness of the antibody was tested through western blot analysis and immunostaining. Samples from 3rd instar larvae that

were ubiquitously expressing *tra*^{f}*-GFP* were prepared for a western blot analysis and probed with our antibody. The antibody failed to detect a signal that could be attributed to the Tra protein (results not shown). Similarly, imaginal wing discs from 3rd instar larvae expressing *tra*^{f}*-GFP* were fixed and incubated in our antibody. *Tra*^{f}*-GFP* expression was driven by the *en-gal4*, which restricts the expression onto one longitudinal half of the wing disc. This time around the antibody did produce a signal. However, we expected a stronger signal on the posterior wing disc but instead the signal was uniform, suggesting that it was non-specific (results not shown). For both, the western and the immunostaining, GFP conjugated Tra^{f} was used as a control. We could probe for GFP with an anti-GFP antibody and confirm presence of Tra^{f}.

Failure of the antibody to detect the Tra protein led us to abandon this track in favor of the second approach, where a tagged version of Tra^{f} was created. This Tra^{f}, containing the Flag tag, was cloned into the pCaSpeR-HS plasmid. This plasmid contains the regulatory region of the *hsp-83* gene that is inserted between the EcoRI and the PstI sites (Thummel & Pirrotta, 1992). The heat shock promoter of the pCaSpeR-HS allows for variable expression depending on the temperature. For *Drosophila*, low expression levels can be obtained at 18°C, intermediate levels of expression can be obtained at 22°C and highest levels can be obtained at 25°C. The highest expression possible with the *hsp-83* is at 29°C.

This HS-Flag-Tra^{f} was utilized to test the idea that Cort acts at the level of Tra in the *Drosophila* sex determination pathway. A western analysis was performed, probing for the Flag tag. Flies were generated that would coexpress both *HA-cort* and *HS-flag-tra*^{f}. The expression of *HA-cort* was driven by *da-Gal4*. Since APC/C targets substances for destruction, we predicted that in the presence of Cort, Tra^{f} levels would decrease. Our western results show exactly this (Figure 2.5, figure S1). *HS-flag-tra*^{f} alone produces a band at around 35 kDa (Figure 2.5 lane 1). When *HA-cort* is coexpressed with *HS-flag-tra*^{f} this band undetectable (Figure 2.5 lane 2). The reduction of this 35 kDa *HS-flag-tra*^{f} band cannot be due to the *da-Gal4* driver, as *HS-flag-tra*^{f} together with *da-Gal4* still produces the characteristic band (Figure S1). Quantification of protein levels from westerns showed significant decrease of Tra levels when *HS-flag-tra*^{f} is coexpressed with *HA-cort* (Figure 2.5 graph).

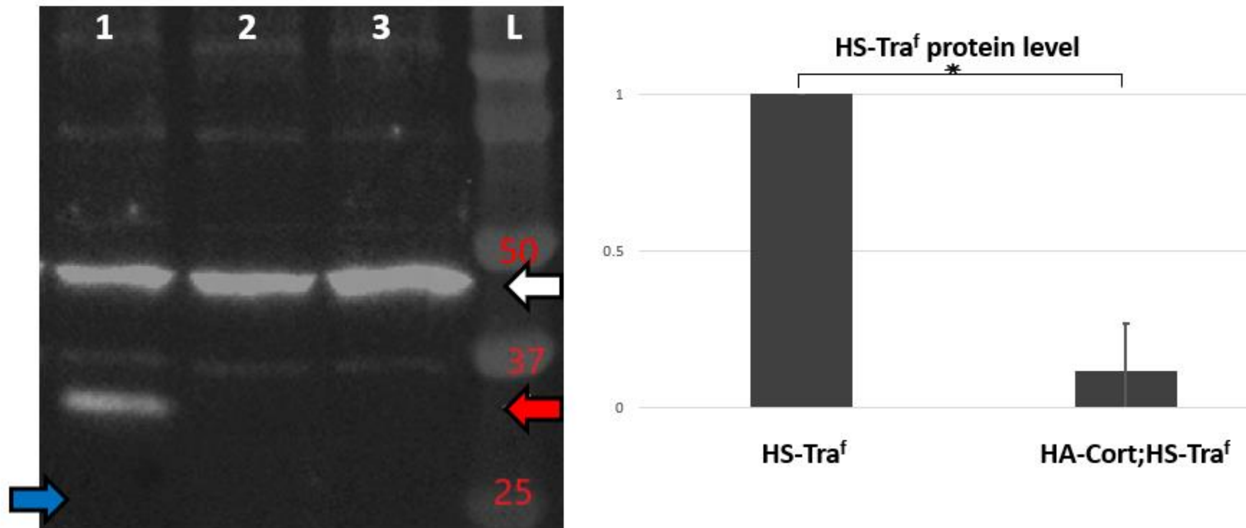

Figure 2.5. In the presence of HA-Cort, HS-Flag-Tra^f levels decrease. Probing for the Flag tag in samples prepared from female 3rd instar larvae resulting from crosses. Red arrow indicate the 35 kDa area on the blot. The white arrow indicates the ~47 kDa protein used for loading correction. Blue arrow indicates the location used to obtain the background noise value (see methods). **(Lane 1)** *HS-flag-tra^f* alone, with expression driven by the heat shock promoter. **(Lane 2)** Coexpression of *UASp-HA-cort^{wt}(9)* (expression driven by *da-Gal4*) and *HS-flag-tra^f*, the HS-flag-Tra^f protein can no longer be detected. **(Lane 3)** female *yw* wildtype control, shows no detectable band at 35 kDa. In other trials (Figure S1), HS-Flag-Tra^f was detectable in the presence of HA-Cort, though its levels were always greatly reduced; unlike this present blot where it is undetectable. The graph shows the quantification of protein levels from 3 different trials (and protein extractions). After removing background noise, the protein levels from *HS-flag-Tra^f* alone were normalized to 1, a ~47 kDa protein was used as loading control. Coexpression of *HA-cort* with *HS-flag-tra^f* leads to a 90% reduction in HS-Flag-Tra^f levels (* = $p<0.05$).

Maternal effects of Cort and regulation of zygotic sex determination

Cort expression in the female germ line is under strict control. Once its purpose is fulfilled it is promptly destroyed in the early embryo (Pesin & Orr-Weaver, 2007). Our results so far show that zygotic misexpression of *cort* causes a sex transformation phenotype in females, likely due to APC/C^{Cort} targeting Tra for destruction. In order to give more context to the finding described by Siera and Cline, (2008) that Tra has a weak ability to initiate the *Sxl* positive feedback loop, we extended our results onto the germline and investigate if a loss of maternal

cort affects sex determination. Complete removal of Cort from an individual causes meiosis to arrest. However, the $Cort^{IR}$ version of Cort imparts the ability to, at low frequency, overcome this terminal meiosis arrest. To this end, *cort* homozygous mutant flies ($cort^{-/-}$) containing a copy of $HA\text{-}cort^{IR}$ with expression driven by the maternal driver *nanos-Gal4* were generated ($cort^{RH}/cort^{QW};nanos\text{-}Gal4/HA\text{-}cort^{IR}$). These females were crossed with male flies that had a duplication for *Sxl* ($Dp(1;Y)Sxl^{+}$). With these conditions, Batiha (2013) noticed that some of the male progeny displayed abnormal external genitalia and analia. Based on these previous findings we predicted that the activity of maternal Tra must be closely monitored to prevent it from activating the *Sxl* positive feedback loop in male progeny. This responsibility lands onto maternal Cort, which allows APC/C to degrade maternal Tra. Sxl production in males could be detrimental for male viability and sexual morphology. Here we repeat the experimental design to test our prediction that loss of cort maternally leads to reduced viability of the male progeny.

To set benchmark numbers for male viability, a control cross was set up, where *cort* heterozygous mutants ($cort^{+/-}$) ($cort^{-}/CyO;nanos\text{-}Gal4/HA\text{-}cort^{IR}$) were used. Like the experimental cross, the $cort^{+/-}$ females were crossed with males with a *Sxl* duplication ($Dp(1;Y)Sxl^{+}$), and the resulting progeny were counted. These counts became the number of males we expect to emerge. The number of males resulting from the experimental cross was compared to this number.

Our model predicts that the consequence of removing maternal Cort will the inappropriate expression of Sxl in males. Sxl is lethal to males and its activation should result in reduced male viability. Under this sensitized genetic background, we found that the removal of Cort did produce an affect on male progeny, with almost a 20% reduction in male viability (Figure 2.6). However, it should be noted that this reduction in male viability is only a trend, as the results are not statistically significant ($p>0.05$). Further repeats of this experiment might provide us with statistically significant and accurate results. In addition to being less viable, these males on some occasions had abnormal genitalia and analia, and very rarely had completely missing genitalia and analia (Figure 1.5 compare B to D, E and F) (Batiha, 2013). These abnormal males accounted for about 10% of the males that reached adulthood (Figure 2.6). The lack of functional external genitalia and analia appears to cause a notable reduction in lifespan (data not shown).

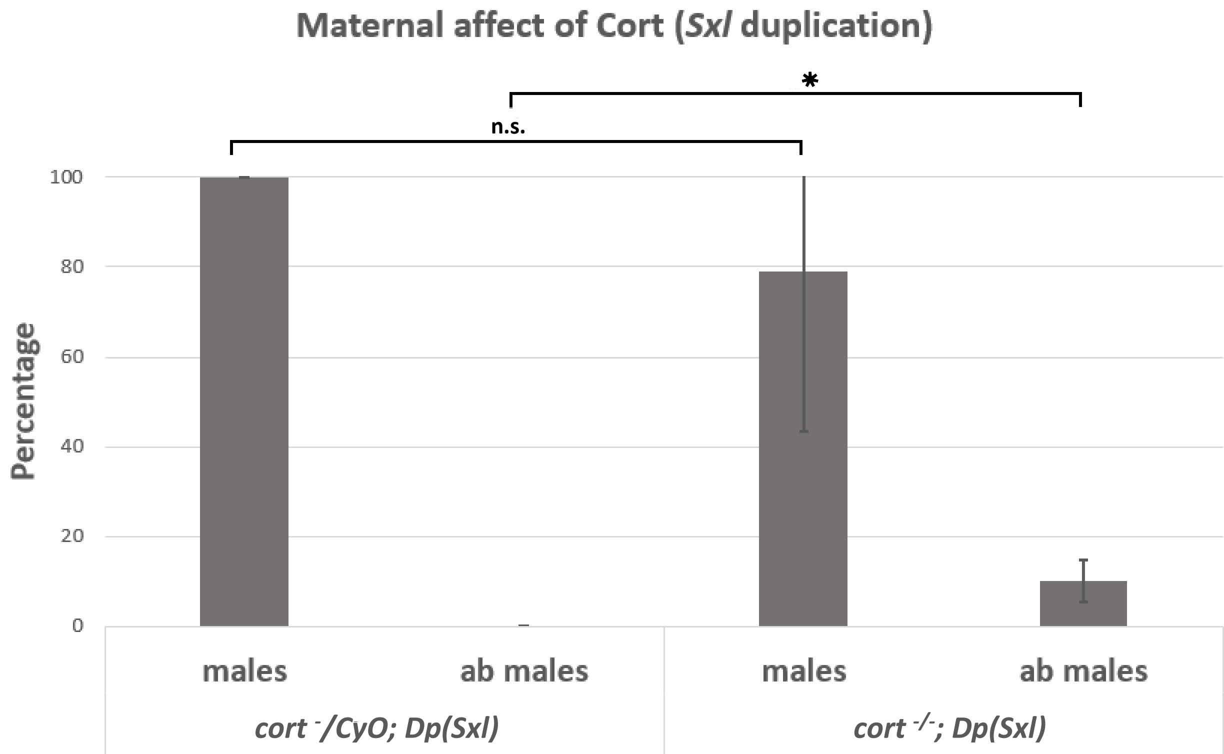

Figure 2.6. *cort* homozygous mutants (*cort^{RH}/cort^{QW};nanos-Gal4/UASp-HA-cort^{IR}*) crossed to *Sxl* duplication (*Dp(1;Y)Sxl^{+}*) (ab = abnormal). In this sensitized background we see almost a 20% reduction in male viability, though these findings are not significant. The male progeny are plagued with defects, with about 10% (n = 10) of them being highly abnormal, having completely missing genitalia and analia. A total of 144 control *cort $^{-}$/CyO* males and 101 *cort$^{-/-}$* males were examined from 4 separate trials, with data analysis done using a T-Test (* = $p<0.05$).

We naturally started our investigation into the maternal loss of *cort* with *Sxl*, since the *Sxl* pre-mRNA has binding sites for Tra (Siera & Cline, 2008). However, it is entirely possible that elements other than Tra exist in the library of Cort targets that could be responsible for the results we are seeing. *SisterlessB* (*sisB* or *scute*) and *sisterlessA* (*sisA*) can be thought to exist on a rung lower than *Sxl* on the ladder of *Drosophila* sex determination. As XSEs, these transcription factors are among the earliest elements to be directly transcribed from the X chromosome and are responsible for the transcription and activation of *Sxl* (Salz & Erickson, 2010). APC/C^{Cort} mediated destruction might act to counteract the build up of XSEs to the point where they could overcome the inhibition threshold setup by Gro/Dpn leading to *Sxl* activation.

Thus, we predict that in a sisA or sisB sensitized background a loss of cort could result in decrease male viability and defects in the sexual morphology of the male progeny. The reduced viability and more strikingly, male-specific abnormalities in external genitalia and analia were previously seen in a *sisA*, *sisB* and/or *Sxl* duplication backgrounds, where these combined duplications were believed to cause the inappropriate *SxlPe* transcription (Cline, 1988).

To determine if the phenotypes we see with loss of cort can be enhanced by a SisA or SisB sensitized genetic background, flies were obtained that had a duplication for either *sisB* or *sisA*. As before, male flies from these stocks were crossed with flies that were *cort* homozygous mutants ($cort^{RH}/cort^{QW}$; *nanos-Gal4/HA-*$cort^{IR}$). For control, the flies used were *cort* heterozygous mutants instead ($cort^{-}$*/CyO;nanos-Gal4/HA-*$cort^{IR}$).

Loss of *cort* in the presence of an extra copy of *sisB* did result in an affect on male morphology. About 31% of the $cort^{-/-}$ male progeny were abnormal, with about 13% appearing to be highly abnormal, having a complete lack of external genitalia and analia (Figure 2.7 middle column and Figure 2.8). However, abnormal males were observed in all conditions of this experiment, with about 2.5% of the male progeny from the control $cort^{+/-}$ being abnormal and about 7% of male progeny that were only *sisB* duplication were abnormal. Both of these conditions did not produce any highly abnormal males that lacked external genitalia and analia (Figure 2.8 left and right column). Unfortunately, we were unable to determine the effect on male viability for our experiments. The *sisB* duplication stock we received were not homozygous. This meant for our control cross we could not differentiate fly genotypes of the progeny with certainty and were unable to establish benchmark numbers of expected males. However, and this should be taken with a grain of salt, from the 3 trials conducted, the experimental cross produced less males (n = 94) than the control cross (n = 134).

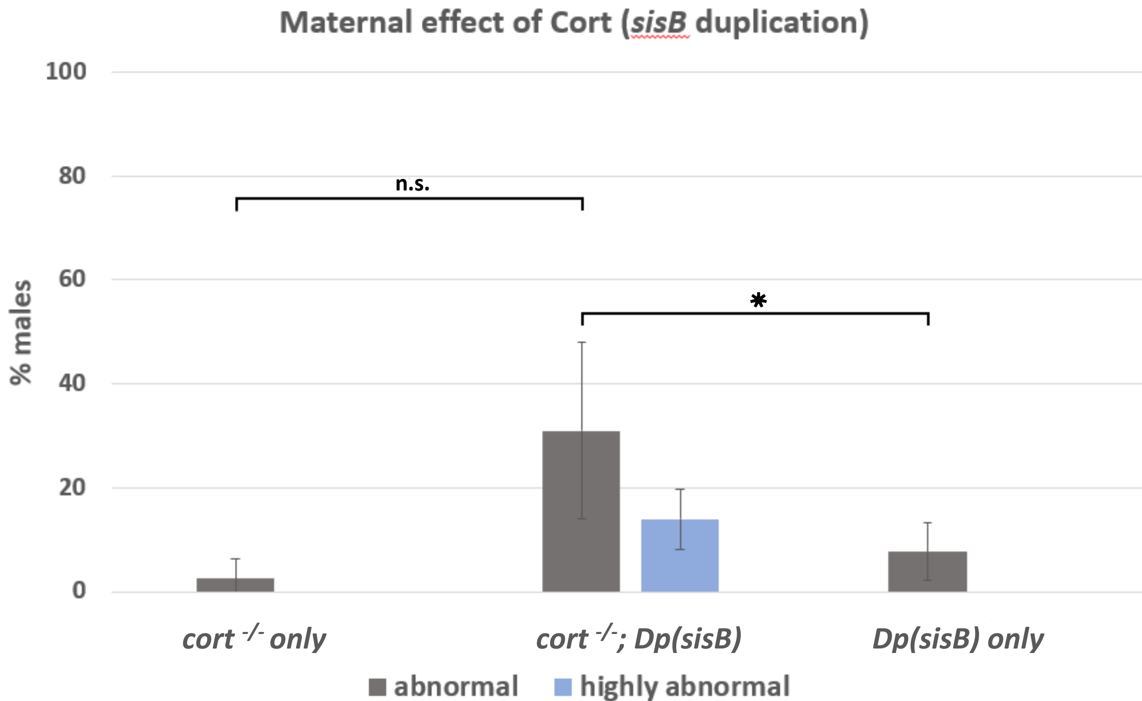

Figure 2.7. Maternal effect of Cort in a *sisB* duplication sensitized background. For the experimental cross females *cort* homozygous mutants (*cort*^{RH}/*cort*^{QW}*; nanos-Gal4/UASp-HA-cort*^{IR}) were crossed to males with *sisB* duplication (*Dp(1;2)sisB*$^{+}$*/CyO*). The control cross was set up using female *cort* heterozygous mutants (*cort* $^{-}$*/CyO; nanos-Gal4/UASp-HA-cort*^{IR}) instead, this is from where we also get the *Dp(sisB)* only progeny (*Dp(1;2)sisB*$^{+}$*/CyO*). Abnormal males with underdeveloped male genitalia and analia were found in both control (2.5%) and experimental crosses (31%). About 7% of male progeny that just had a *sisB* duplication were abnormal. Only the experimental cross produced males that were highly abnormal, complete lack of external genitalia and analia (13%). A total of 134 control *cort*$^{-/}$*CyO* males and 94 *cort*$^{-/-}$ males were examined with data analysis done using a T-test (* = $p<0.05$).

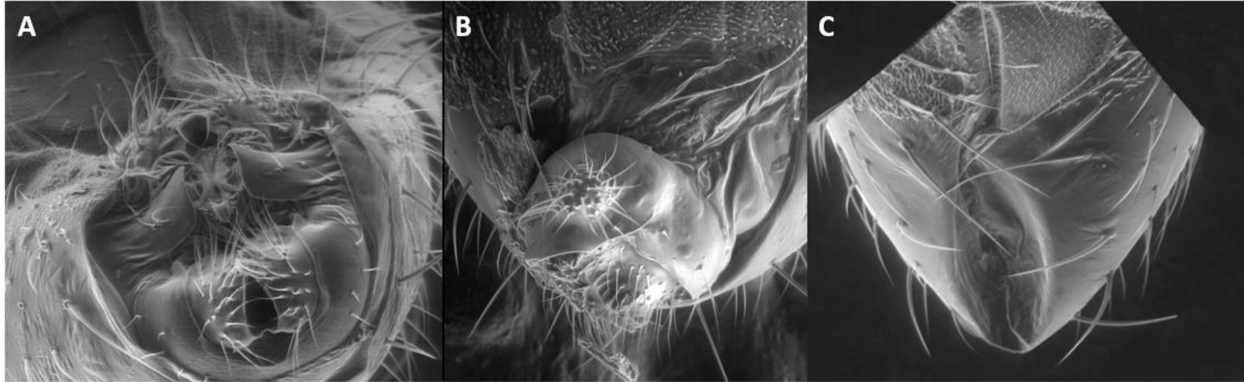

Figure 2.8. **(A)** A wildtype male showing normal genitalia and analia. **(B and C)** various degree of abnormal male development, abnormal genitalia seen in some of the male progeny from *cort* homozygous mutants (*cort*^{RH}/*cort*^{QW};*nanos-Gal4/UASp-HA-cort*^{IR}) crossed to *sisB* duplication (*Dp(1;2)sisB*$^{+}$/*CyO*). **(B)** an example of *XY;cort* $^{-/-}$ x *sisB*$^{+}$*Dp* showing abnormal genitalia and analia. **(C)** an example of *XY;cort* $^{-/-}$ x *sisB*$^{+}$*Dp* showing highly abnormal genitalia and analia.

Due to technical problems, we were unable to assess male viability in *cort* homozygous mutant with *sisB* duplication, but we were able to do it in *sisA* duplication. Same procedure was followed as with *sisB* experiments. Female *cort* homozygous mutants (*cort*^{RH}/*cort*^{QW}*; nanos-Gal4/UASp-HA-cort*^{IR}) were crossed with males that had a *sisA* duplication (*Dp(1;2)sisA*$^{+}$). To establish a benchmark number for expected number of males, a control cross was set up where female *cort* heterozygous mutants (*cort* $^{-}$/*CyO; nanos-Gal4/UASp-HA-cort*^{IR}) were used. Under these conditions male viability dropped to around 68%; however, no abnormal males were detected among the progeny. Unfortunately, these results are preliminary and require further investigation and repeats (Figure 2.10). With our results showing the enhancement of the loss of maternal *cort* phenotype in a *sisB*/*sisA* and *Sxl* sensitized backgrounds, it is possible that Cort might be affecting *SxlPe* transcription rather than or perhaps in addition to affecting maternal Tra.

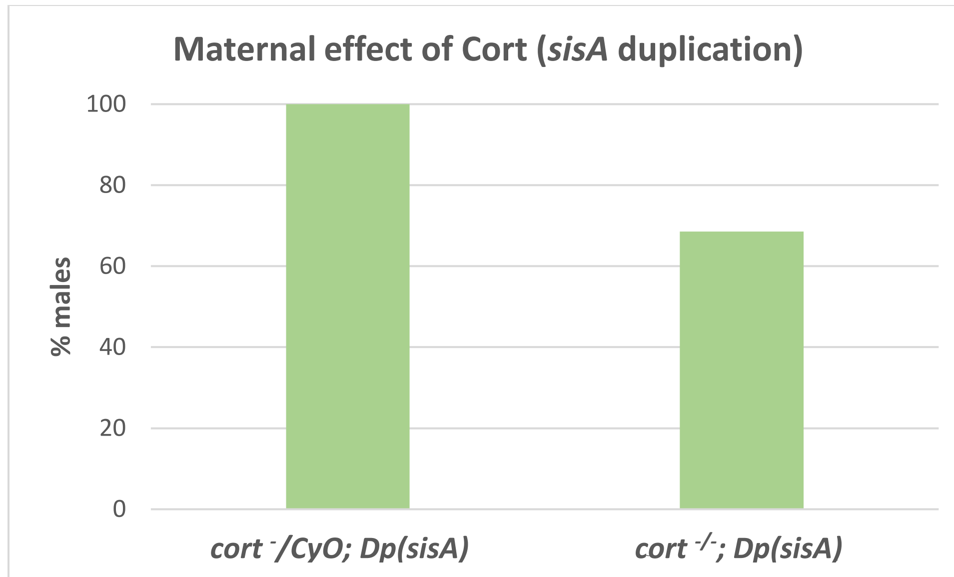

Figure 2.9. Preliminary results for maternal effect of maternal Cort in a *sisA* duplication sensitized background. **(cort $^{-}$/CyO;Dp(sisA))** Control *Cort* heterozygous mutants (*cort $^{-}$/CyO;nanos-Gal4/UASp-HA-cort^{IR}*) crossed to *sisA* duplication (*Dp(1;2)sisA^{+}*), the number of resulting male progeny formed the % of males expected. **(Cort $^{-/-}$, Dp(sisA))** *cort* homozygous mutants (*cort^{RH}/cort^{QW};nanos-Gal4/UASp-HA-cort^{IR}*) crossed to *sisA* duplication (*Dp(1;2)sisA^{+}*). There was a 32% reduction in male viability. Statistical analysis not done; data from only a single trial is displayed.

Batiha (2013) RT-PCR analysis showed that the abnormal male progeny had both the male and female isoform of *Sxl.* In order to confirm these previous findings plans were in place to repeat this investigation into the loss of maternal Cort using q-RT-PCR. Analysis was being performed on cDNA samples from the abnormal male progeny of *cort* homozygous mutant mothers. Primers against *Sxl*, *dsx* and *tra* were designed and made, but unfortunately the results proved to be highly inconsistent, stemming from the lack of cooperation from the primers. Perhaps the premiers need to be re-made or the PCR conditions need to be fine tuned.

In summary, through genetic epistasis experiments we were able to confirm that *cort* acts on the level of *tra*, and that *cort* is partially epistatic to *tra*. We also confirmed that the sex transformation phenotype associated with *cort* misexpression is a result of Cort and APC/C interaction. More importantly, our western analysis showed that in presence of Cort there is a significant reduction of Tra^{f}.

In exploration into the maternal role of Cort we saw a trend where loss of maternal *cort* leads to lower male viability in *Sxl* and *sisA* sensitized backgrounds; in *Sxl* and *sisB* sensitized backgrounds loss of maternal *cort* produced an effect on male sexual morphology, where some of the progeny had abnormal genitalia and analia.

CHAPTER III: DISCUSSION

Cort acts on Tra in an APC/C dependent manner

The continuation of life hinges on the cell cycle, making it one of the most important biological processes on the planet. The Anaphase Promoting Complex/Cyclosome and its co-activators are at the center of it all, playing vital roles in both meiosis and mitosis. Thus, a complete understanding of how the APC/C and its co-activators fit into the complex mechanisms of the cell cycle holds great value.

Cort is a distant relative of the APC/C activators, Fzy and Fzr. It only appears in *Drosophila* female meiosis and is a pivotal cell cycle regulator in both of its divisions. *Cort* mutants arrest before the completion of meiosis as Cort is prerequisite for the destruction of cyclins and Securin (Pesin & Orr-Weaver, 2007; Swan & Schüpbach, 2007). Cort also plays a part in the oocyte to embryo transition, that includes, in addition to meiosis completion, translational and polyadenylation control of early embryonic patterning genes (*bicoid* and *toll*), transcript destabilization and microtubule reorganization (Lieberfarb et al., 1996; Tadros et al., 2003). These multifaceted roles of Cort are still a part of ongoing investigations and is also the purpose of this study.

Governance over Cort expression at the transcriptional, translational and protein stability level is extremely strict; with *cort* being expressed late oogenesis. Its mRNA is deadenylated and repressed in early-stage embryos, and Cort protein is promptly removed two hours into embryogenesis (Pesin & Orr-Weaver, 2007). Cort misexpression, outside of this restricted expression in the germline, produces a gain-of-function phenotype where it affects the female sexual development and transforms genetically female individuals to male-like individuals.

These transformed females retain the larger size, typical of wildtype females, but also gain male characteristics like pigmentation on abdomen, sex combs and male-like genitalia (Batiha, 2013). Examination into the internal structures of individuals transformed via Cort misexpression revealed them to resemble male testes (Figure 2.1). Naturally the transformed internal and external structures make these individuals sterile.

Batiha (2013) showed *cort* misexpression causes the sex transformation due to its interference in the sex determination pathway somewhere downstream of *Sxl* splicing and upstream of *dsx* and *fru* splicing. This led to the idea that Cort disrupts *tra* expression. We used the ability of the sex transformation phenotype to affect the sex combs to establish the relationship between Cort and Tra. Examining the sex combs proved itself to be a useful tool as it allows for easy quantification of phenotypes. Ubiquitous expression of *HA-cort* (driven by *da-Gal4*) showed that HA-Cort retained most of its capacity to induce the transformation phenotype in the presence of Tra^f (Figure 2.3), which means that Cort is partially epistatic to Tra. Hypothetically, if Cort were to impede Tra activity indirectly by supressing Sxl activity, then we would see a complete epistasis of tra^f over *cort*. If that were true, we would see a male transformation to females. However, this is not the case as we are seeing an incomplete transformation of females to males. Conversely, if Cort were to interfere with *dsx*, we would not see a sex transformation phenotype in male flies expressing both HA-Cort and Tra^f; instead, there would be an incomplete development of the genitalia or a lower sex comb scores for males (females will not develop sex combs). However, if Cort restricted activities of the Tra protein, then Cort epistasis over Tra^f would depend on Tra levels. The *HS-flag-*tra^f insert employed for this analysis contains a strong *HSP-83* promoter that permits high levels of expression, though not as strong as *UAS* promoters. Since Cort driven transformation is still seen in the presence of high levels of Tra^f, it implies that *cort* interferes with *tra* post splicing. The cause of the partial epitasis we witness could be rooted in gene expression. As mentioned earlier the *HSP-83* is not a weak promoter. Add to that the endogenous levels of Tra and we could find a situation where there isn't much Cort to fully cull Tra activity. This could be further explored using genetic experiments where we decrease the gene dose for *tra* and see how it affects the degree of transformation.

Cort is an APC/C activator and APC/C is a ubiquitin ligase; thus it is easy to predict that Cort functions by inhibiting Tra as part of the APC/C. The *HA-cort*^{IR} transgene, a version of *HA-cort* that lacks the IR tail motif, allows for the opportunity to test this prediction. The IR tail is a conserved motif that is part of all APC/C coactivators; it enables the interaction of the coactivators to an APC/C subunit, the Cdc23 (Chu et al., 2001). HA-Cort^{IR} is unable to induce the transformation phenotype (Batiha, 2013). Coexpression of *tra*^{f} and *HA-Cort*^{IR} leads to the loss of partial epistasis seen in *tra-GFP/HA-cort*, with the resulting progeny having a male to female transformation (Figure 2.3). Approaching this idea from a different direction also produced the same conclusion. Instead of making Cort ineffective in the APC/C^{Cort} complex, we made the APC/C inoperative. Misexpression of *cort* in individuals that had RNAi against *cdc23* failed to produce the sex transformation phenotype (Figure 2.4).

These two pieces of evidence allow us to speculate with high certainty that the ability of Cort to influence female sexual morphology is due to its activity in cooperation with APC/C. APC/C^{Cort} could potentially affect sexual morphology by ubiquitinating some protein in the sex determination pathway. Since Cort acts at the level of Tra, we reason that the particular protein targeted by APC/C^{Cort} to be the Tra protein. This could be proven by directly looking at Tra levels in presence of Cort. In pursuit of this evidence, we generated a novel anti Tra antibody; however, we were unsuccessful in getting that antibody to detect Tra in immunostaining and western blot analysis. Thus, we opted for the indirect approach using a tagged version of Tra. The HS-Flag-Tra^{f} transgene proved to be a suitable alternative. This fusion protein produced a detectable band at around 35 kDa; and indeed, in the presence of HA-Cort, the HS-Flag-Tra^{f} band was greatly diminished (Figure 2.5). This lowering of HS-Flag-Tra^{f} levels is without doubt associated with its coexpression with HA-Cort, as other conditions where HA-Cort was not involved (*HS-flag-tra*^{f} *x da-Gal4*) still produced the HS-Flag-Tra^{f} band (Figure S1).

Maternal role of APC/C^{Cort} and its regulation of zygotic sex determination

For a while there was an understanding that the maternal Tra had no function in the female germline. Even though Tra is expressed in the female germline, a loss of *tra* had no consequences, not even affecting sex determination in the female germline (Marsh & Wieschaus,

1978). Siera and Cline (2008) uncovered that Tra can activate the *Sxl* feedback loop by binding to the *Sxl* pre mRNA and causing female specific splicing, albeit this quirk of Tra is considerably weak. Going off their findings we try to give context to APC/C^{Cort} mediated destruction of Tra, i.e., a consequence of losing maternal *cort* is the deleterious effects on the development of male progeny due to activation of *Sxl*.

Our results conform to this idea, however the statistical analysis does not; the reduction in male viability was found to not be significant ($p<0.05$). In sensitized genetic backgrounds of *Sxl* duplication, *cort* homozygous mutants had reduced numbers of viable male progeny (Figure 2.6). Additionally, some of the male progeny had abnormal genitalia and analia, and on rare occasion completely lacked genitalia and analia. These abnormal males also suffered from sterility and reduced lifespans (data not shown). This contrasts with zygotic misexpression of *cort* which results in transformation of females to male-like fates; likely through APC/C^{Cort} mediated destruction of Tra. Based on those results, we reasoned for a necessity of Cort targeting Tra in the oocyte or early embryo which serves to prevent activation of *Sxl* in males.

Tra in the oocyte acts on the *Sxl* pre-mRNA, owing to the multiple Tra binding sites present on the *Sxl* pre-mRNA, thus producing a functional Sxl. Therefore, we theorize that excess Tra (that accumulates in the absence of Cort) initiates the *Sxl* positive feedback loop, since one of the targets of Sxl is its own mRNA. Presence of functional Sxl in males could disrupt the production of the Msl-2 protein. Since Sxl prevents Msl-2 assembly in two ways, through intron retention in the nucleus and translation inhibition in the cytoplasm (Gebauer et al., 1998; Beckmann et al., 2005), even a slight presence of Sxl could majorly disrupt Msl-2 production. This might hinder male development in some cases and cause lethality in others

Our results from zygotic misexpression of Cort painted Tra as its likely target. Coupled with the fact that *Sxl* pre-mRNA has Tra binding sites; we predict in our loss of maternal *cort* model that the activation of *Sxl* could be due to unchecked maternal Tra activity. However, it would be interesting to investigate different possibilities or other unknown Cort targets. The XSEs responsible for *Sxl* activation is a good place to start. When repeating the loss of maternal *cort* experimental design in a *sisA* duplication sensitized background, we saw a trend towards a reduction of male viability; although, we failed to see any abnormal males. Furthermore, a loss of maternal *cort* in a *sisB* duplication sensitized background produced a greater number of

abnormal males compared to *Sxl* duplication. Cline (1988) reported on how duplication of *Sxl* with duplication in *sisA* or *sisB* resulted in the strange phenotype where males had reduced viability and morphological deficits. This was interpreted as an inappropriate transcription of the *SxlPe*, which results in the production *Sxl* and activation of the *Sxl* positive feedback loop in males. The preliminary results presented here display the same phenotype, which at least implies the loss of maternal Cort seems to affect *SxlPe* transcription.

APC/C^{Cort} regulation of Tra might have evolutionary implications

In other Diptera species *tra* occupies the highest seat on the sex determination pathway, with *Drosophila* being the only known exception, where *Sxl* is the master regulator (Verhulst et al., 2010). In female Diptera, *tra* participates in an autoregulatory loop involving its own mRNA to bring about its productive splicing to the female isoform. In these Diptera, maternally deposited *tra* aids in establishing the autoregulatory feedback loop that leads to female development. The divergence also extends to the Y-chromosome; in some Diptera the Y-chromosome produces masculizing (M) factor that obstruct the transcription or translation of *tra* preventing the autoregulatory loop from being established. On the other hand, the Y-chromosome in *Drosophila* is not involved in sex determination whatsoever (Verhulst et al., 2010). Thus, the ability of the Diptera Y-chromosome to dissuade feminization of males does not exist in *Drosophila* males. This is where we speculate the role of maternal Cort targeting maternal Tra could be, a potential safeguard against maternal Tra feminizing *Drosophila* males. This idea can be extrapolated onto other Dipteras, where maternal Cort provides a safety net of sorts, in case the Y-chromosome fails to antagonize Tra.

Sxl being the master regulatory gene in a cascade is only observed in *Drosophila* which probably means that *Sxl* is a recent evolution, with *Tra* being a representation of an ancestral past. With *tra's* status being downgraded in the *Drosophila* sex determination hierarchy, it also lost its ability to autoregulate itself. It is possible that Tra tries to maintain a form of autoregulation through *Sxl*; indeed, functional Tra cannot exist without Sxl bringing about its productive splicing. The answer to this riddle lies in the fact that *Sxl* pre-mRNA has binding sites for Tra. How did these sites end up in *Sxl* pre-mRNA, and why have they been conserved for

millions of years? It is possible that maternal Tra has a yet to be identified role in promoting female fate in progeny and that Cort counteracts it to promote the male fate.

Future directions

Regarding the investigations into the internal workings of transformed individuals, we can conduct a behavioral analysis on adult flies. Fru oversees the developmental aspects of sexual behaviors and orientation. Tra and its partner Tra-2 directly control *fru* splicing and female specific splicing of *fru*. *Fru* is instrumental in providing males with the ability to recognize females as suitable courtship partners (Ryner et al., 1996). As these transformed individuals contain both isoforms of *fru*, it would be interesting to see what kind of courtship behavior they display, or whether they can discriminate between male and females when it comes to courtship. This line of investigation would require camera setups to monitor courtship behaviours adults sex transformed flies and compare them to wildtype males.

In order to solidify the necessity of APC/C in the sex transformation, additional investigation into the role of Cort's C-box is warranted. The C-box is another motif present on the N-terminus of Cort that mediates its interaction with the APC/C, in addition to the IR tail. In preliminary experiments, we made C-box mutants ($HA\text{-}cort^{C\text{-}box}$). Multiple lines of these mutant flies were tested for their ability to cause sex transformation. All the lines tested did fail to produce the sex transformation (A.S. unpublished data). $HA\text{-}cort^{IR}$ was used as a control, as it also fails to cause sex transformation. Unfortunately, due to time constraints we were unable to quantify these results or verify $HA\text{-}cort^{C\text{-}box}$ expression, is on the same level as $HA\text{-}cort^{wt}$ expression.

To further strengthen our results that APC/C^{Cort} targets Tra for destruction we could investigate the 26S proteosome pathway. By using labeled ubiquitin we can track ubiquitination of Tra protein. Moreover, adding a proteosome inhibitor and checking to see if we can see stabilized Tra protein in assays should be solid evidence. Plans to conduct these experiments in S2 *Drosophila* cell cultures were underway. Attempts to make constructs for tagged *cort*, $cort^{C\text{-}box}$, *fzy* and *tra* were made.

Several future directions were conceived for the role of maternal Cort. Since we saw that loss of cort phenotype was enhanced by a *sisB* duplication which suggests an effect on *Sxl* transcription, other transcription factors of *Sxl* are worth investigating with Daughterless (Da) being a good candidate, which like *cort*, is also maternal. Da forms a heterodimer with sisB forming a DNA binding complex that acts on the *Sxl* early promoter (Yang et al., 2001). If we observe a reduction in viability and defects in the sexual morphology of males in a maternal Da duplication sensitized background, then the suspicion falls onto the XSEs themselves. Maternal Cort could be tasked with cleaning up the excess XSEs, preventing them from accumulating to the point where they start to overcome the Gro-Dpn mediated repression of *Sxl*.

An *in situ* hybridization experiment can be used to check for if the loss of maternal Cort does indeed lead to *SxlPe* expression in male embryos. 0-2h male embryos collected from a *cort*$^{-/-}$ x *Dp*(*Sxl*$^{+}$) cross can be subjected to probes that specifically detect the female *Sxl* mRNA. If our model is correct, we should see a signal from *Sxl* transcription in these early embryos.

Our inquiry into the maternal role of *cort* is based on the extrapolation of our finding of Cort mediated Tra's destruction in the zygote. However, we don't know for certain if this holds true in the egg. By using maternal drivers (like *mat67-Gal4*), an experimental design similar to that of figure 2.5 can be repeated in the germ line. Limiting *cort* expression to germ line will allow us to quantify Tra levels in the presence of Cort in the eggs laid.

Conclusion

In this thesis, I investigate Cort, the *Drosophila* female specific meiosis activator of the APC/C and the role it plays in *Drosophila* sex determination. APC/C is a ubiquitin ligase employed by the cell to exercise control over the cell cycle. Its most pivotal role is the faithful segregation of sister chromatids during metaphase II and ushering in anaphase. *Cort* is one of many maternally deposited mRNAs, becoming active in oocyte stage 14 and remaining active for about 2 hour into embryogenesis. *Cort* is necessary for the completion of meiosis in females, as *cort* mutants are known to arrest in metaphase II (Lieberfarb et al., 1996; Swan & Schüpbach, 2007). Zygotic misexpression of *cort* outside of its strict window has highly adverse

consequences on sexual development, transforming genetically female individuals to male-like individuals. Evidence presented in this thesis supports a model in which Cort acts at the level of *tra* in the sex determination pathway; likely through APC/C^{Cort} mediated ubiquitination of Tra and its subsequent destruction via 26S proteosome.

Tra has the capacity to weakly initiate the *Sxl* positive feedback loop, owing to its ability to act on *Sxl* pre-mRNA (Siera & Cline, 2008). Our results suggesting that Cort targets Tra zygotically gave us a reason to extend this interaction to the maternal germ line. We theorize that maternally deposited Cort targets maternally deposited Tra to prevent the activation of the *Sxl* positive feedback loop in male embryos. In genetically sensitized backgrounds, we found evidence for developmental abnormalities and reduction of male viability in progeny of mothers that were *cort* homozygous mutants. I found that *sisB* duplication can enhance this loss of *cort* phenotype suggesting that cort is involved in the transcription of *SxlPe.* With *Sxl* pre-mRNA having Tra binding sites, future work will be needed to determine if maternal Cort acts on Tra, or on one of the XSEs, or possibly on both. Our findings on the loss of maternal Cort has evolutionary implications; especially in Diptera, where *tra* is the master sex regulator, instead of *Sxl*. In Diptera, maternally deposited Tra is responsible for activation of its own self regulatory positive feedback loop (Ruiz et al., 2007); we speculate that maternal Cort is tasked with keeping maternal Tra from activating its own feedback loop in males, providing a secondary layer of defence against feminization of males.

CHAPTER IV: MATERIALS AND METHODS

Stocks and crosses for HA-Cort and Tra^{f}-GFP epistasis

The following stocks were used for to develop the HA-Cort and Tra^{f}-GFP epistatic relationship:

Table 4.1. Stocks used for Figure 2.3, *cort-tra* epistasis.

Stock	Source	Notes
UASp-GFP;UASp-HA-cort^{wt}*(3,7)*	Batiha, 2013	Insertion line for *cort*^{wt}
UASp-tra^{f}*-GFP;UASp-HA-cort*^{wt}*(3,7)*	Batiha, 2013	Insertion line for *Tra*^{f}*-GFP* and *cort*^{wt}
UASp-tra^{f}*-GFP;UASp-HA-cort*^{IR}	Batiha, 2013	Insertion line for *tra*^{f}*-GFP* and *cort*^{IR}
yw;da-Gal4/da-Gal4	BDSC	A universal Gal4 driver
yw	BDSC	Flies with Yellow/white markers, serve as wildtype control

Markers (*Star* and *Pr*) and balancers (*CyO* and *Tm6*) were used to identify genotypes. The progeny from each cross were collected and their sex combs quantified. To obtain flies that were co-expressing both *HA-cort* and *tra*^{f}, males from *UASp-tra*^{f}*-GFP;UASp-HA-cort*^{wt}*(3,7)* flies were crossed to females from *yw;da-Gal4*. To see the effect of *cort* alone male *UASp-GFP;HA-cort*^{wt}*(3,7)* were crossed to females from *yw;da-Gal4*. To see the effect of *tra* expression in presence of impaired *cort*, *UASp-tra*^{f}*-GFP;UASp-HA-cort*^{IR} were crossed to females from *yw;da-Gal4*. The *UAS-GFP* stock was used to ensure all crosses had the number of *UAS* elements balanced out.

Stocks and crosses for Cdc23RNAi and HA-Cort epistasis

The following stocks were used to study the role of APC/C subunit cdc23 in sex transformation:

Table 4.2. Stocks used for Figure 2.4, role of APC/C subunit Cdc23

Stock	**Source**	**Notes**
UASp-cdc23^{RNAi}	Stock 5280 - VDRC	Insertion line for *cdc23^{RNAi}*
UASp-HA-cort^{wt}(3,7)	BDSC	Insertion line for *cort^{wt}*
UASp-cdc23^{RNAi};UASp-HA-cort^{wt}(3,7)	A.S. unpublished	Generated by crossing *UASp-cdc23^{RNAi}* with *USAp-HA-cort^{wt} (3,7)*
yw	BDSC	Flies with *yellow/white* markers
rn-gal4	BDSC	A Gal4 driver that limits expression to the legs

BDSC = Bloomington *Drosophila* Stock Center, VDRC = Vienna *Drosophila* Resource Center.

Rn-gal4 was employed to limit expression to the legs, this limits the transformation phenotype to the legs, thus only effecting the sex combs. Adult progeny from the crosses were captured and their sex combs were quantified. For co-expression of *cdc23^{RNAi}* and *HA-cort*, males from *UASp-cdc23^{RNAi};UASp-HA- cort^{wt}(3,7)* were crossed with *rn-Gal4*. For controls *UASp-cdc23^{RNAi}* and *UASp-HA-cort^{wt}(3,7)* were separately crossed to *rn-Gal4*. These crosses were carried out at 18°C, as *rn-Gal4* works better at lower temperatures, producing sex combs that are easier to identify and quantify. The crosses were initially carried out at 22°C, but the nature of sex combs at this temperature made them extremely difficult to quantify.

Stocks and crosses for HA-cort and HS-Flag-Tra western blot

For western blot analysis on the HA-Cort and HS-Flag-Tra^f interaction, the following stocks were used:

Table 4.3. Stocks used for Figure 2.5, *HA-cort* and *hs-flag-tra^f* western analysis.

Stock	Source	Notes
HS-flag-tra^f(1)	Genome Prolab	Flag tagged *tra^f* construct injected into embryos
HS-flag-tra^f;UASp-HA-$cort^{wt}$(9)	A.S. unpublished	Generated by crossing females from *HS-flag-tra^f(1)* to males from *UASp-HA-$cort^{wt}$(9)*
yw	BDSC	Flies with Yellow/white markers
yw;da-Gal4/ da-Gal4	BDSC	A universal Gal4 driver

Female 3rd instar wandering larvae were collected from each cross. Female *UASp-HA-$cort^{wt}$(9);HS-flag-tra^f(1)* were crossed to male *da-Gal4*. Larvae collected from *yw* served as a negative control. Female larva from *HS-flag-tra^f(1)* stock crossed to *yw* were used to established HS-Flag-Tra^f levels in absence of *cort*. Females from *HS-flag-tra^f(1)* were crossed to males from *yw;da-Gal4* to check for any possible effect caused by *da-Gal4*.

Stocks and crosses for testing the novel Tra antibody

To test our novel Tra antibody the following stocks were used:

Table 4.4. Stocks used to test our novel Tra antibody.

Stock	Source	Notes
UASp-Tra^{f}-GFP	Batiha, 2013	Insertion line for *tra^{f}*
en-Gal4	BDSC	A Gal4 driver that limits expression to a part of imaginal wing disc
yw;da-gal4/da-gal4	BDSC	A ubiquitous Gal4 driver

Our Tra antibody was tested using immunostaining and western blotting. 3rd instar wandering larvae resulting from the crosses were collected and processed. Male *UASp-Tra^{f}-GFP* flies were crossed to female *en-Gal4* at 25°C (for immunostaining) or female *da-Gal4* at 22°C (for western blotting).

Stocks and crosses for maternal effect of Cort

For the investigation into maternal role of Cort the following stocks were used:

Table 4.5. Stocks used for the investigation into the maternal role of *cort*.

Stock	Source	Notes
cort^{RH}	Schüpbach, 1987	Mutant line for *cort*
cort^{QW}	Schüpbach, 1987	Mutant line for *cort*
Dp(1;Y)ct[+]y[+]	BDSC	*Sxl* duplication
Dp(1;2)v[+]65b	BDSC	Homozygous *sisA^{+}* duplication
Dp(1;2)sc19	BDSC	*sisB^{+}* duplication over *CyO* balancer
yw	BDSC	Flies with y*ellow*/*white* markers, serve as wildtype control
nanos-gal4	BDSC	Maternal Gal4 driver

UASp-HA-Cort^{IR} and *nanos-gal4* were added to the two *Cort* mutant stocks (*cort*^{QW} and *cort*^{RH}) to generate *cort*^{QW}*;nanos-Gal4/UASp-HA-cort*^{IR} and *cort*^{RH}*;nanos-Gal4/UASp-HA-cort*^{IR}. These stocks allowed us to circumvent the arrest caused by a loss of *cort*. Flies from these two stocks were crossed to each other to obtain *cort* homozygous mutant flies (*cort*^{RH}*/cort*^{QW}*;nanos-Gal4/UASp-HA-cort*^{IR}) or *cort* heterozygous flies (*cort*^{RH}*/CyO;nanos-Gal4/UASp-HA-cort*^{IR} or *cort*^{QW}*/CyO;nanos-Gal4/UASp-HA-cort*^{IR}). Females from these were subsequently crossed to males from *Sxl*, *sisA* and *sisB* duplication stocks. To set up control conditions, females flies that were *cort* heterozygous were crossed to males from *Sxl, sisA* and *sisB* duplication stocks.

Generation of transgenic flies

The Flag tagged *tra*^{f} insert (~700 bp) was isolated from *UASp-tra*^{f}*-GFP* (Batiha, 2013). The insert was amplified using the following primers that included the restriction sites for EcoRI and XbaI

RI-flag-f: acacGAATTCATGGACTACAAAGACCATGACG (Tm: 74.9°C)

traFem-Xba-r: acacTCTAGATCAATATGGGGACGCCCCGCG (Tm = 82.1°C)

The insert was purified out of the PCR product using the EZ-spin column purification kit (BioBasic) and then digested using the aforementioned enzymes. Digested *flag-tra*^{f} insert was purified using isopropanol precipitation and resuspended in TE. Presence of the insert was confirmed using gel electrophoresis and the appropriate band was extracted out of the agarose gel (Qiagen gel extraction kit cat # 228706).

pCaSpeR-HS was ordered from *Drosophila* Genomic Research Center (DRGC; stock 1215). The plasmid was digested using EcoRI and XbaI enzymes, and plasmid was purified using isopropanol precipitation. The *flag-tra*^{f} insert and the pCaSpeR-HS were ligated using the T4 ligase in 1:3 vector to insert ratio. The ligation was transformed into DH-5 alpha cells (NEB) and the resulting colonies were probed for the insert using colony PCR. Successfully ligated plasmids were isolated using mini-prep kit and sent for sequencing (Sickkids Center for Applied Genomics) to ensure no errors were made during the PCR process. The following primers were used for sequencing:

446-426: GCTCTTTGGCGCAATCTTCTC (Tm = 61.2)

426-446: GAGAAGATTGCGCCAAAGAGC (Tm = 61.2)

The construct was also subjected to a test double digest using HindIII (restriction site in the plasmid) and NotI (restriction site in the *flag-tra^f* insert). Plasmid constructs were then sent for micro-injection into the *Drosophila* genome (Genome Prolab). The adult *HS-flag-tra^f* fly lines that were obtained were tested for expression levels. Western blotting was preformed on third instar larvae, probing for the Flag tag. *HS-flag-tra^f(1)* was chosen for their high expression.

Sex comb quantification and sex comb score

One of the forelegs from an adult fly was dissected out and mounted onto a microscopic slide. The sex comb appears just below the second to last joint on the leg. That area on the leg was examined for each leg on the Leica DMI6000 fluorescent microscope (at 10X, 20X and 40X). Each individual bristle of the sex comb was counted and given a score, if the hair was thick and rounded at the end, it received a score of 1. If the hair was of partial length, it received a score of 0.5. For each individual, the sex comb score was the sum of all bristle scores. For each condition at least 10 legs were collected, and only one leg was taken from an individual. For each epistasis condition 3 separate trials were conducted, and average sex comb score was calculated. A single-tailed T-Test was performed on the 3 average sex comb scores. The error bars are the standard deviation which was calculated from the raw data.

Western blotting

Protein extracts were prepared by collecting and bisecting 3rd instar wandering larvae, turning each bisected half inside out. Samples were either flash frozen in liquid nitrogen or immediately added to 2x sample buffer (30 uL per larva). The sample was immediately ground up, homogenized and boiled for at least 5 minutes at 65°C. The samples were then loaded into a 10% SDS polyacrylamide. The following antibodies were used: rabbit anti-Tra, novel antibody, made by Biomatik from the following peptide sequence: YHGRSSERDSRKKEH-Cys (used in

various concentrations ranging from 1/500 to 1/5000), mouse anti Flag (Flag M2, Sigma-Aldrich) (1/1000), rat anti HA (rat HA 3F10, F. Hoffmann-La Roche AG) (1/1000), rabbit anti-GFP (Torrey Pines GFP, Torrey Pines Biolabs) (1/2000). The following secondary HRP antibodies were used at 1/14000: anti rat HRP, anti mouse HRP and anti rabbit HRP. Each antibody staining was followed by 3 rinses in TBST and 3 10-minute washes in TBST.

Blots were treated with ECL solution (ThermoFisher SuperSignal West Pico PLUS) and imaged using BioRad ChemiDoc imager. Alphaimager Software was used to analyze pixels to determine protein loading. A standard area was defined which was used for each band tested. An area away from the lanes was used to serve as the background; from each band the background noise was subtracted. Then a non-specific band from the same lane was used to normalize experimental band value to obtain the corrected loading. A one-tailed T-Test was performed on the corrected band values obtained from 3 different blots to determine statistical significance

Immunofluorescence

Wing imaginal discs were dissected out of female 3rd instar wandering larva and fixed in 3.5% formaldehyde and PBST for 30 minutes, followed by an incubation in 1% BSA blocking solution for 1 hour. Samples were incubated in Tra antibody (at multiple concentrations: 1/250, 1/500 and 1/1000) overnight at 4°C, followed by an overnight incubation at 4°C in Alexa secondary antibody (Alexa Fluro 568, ThermoFisher) (1/1000). Each antibody staining was followed by 3 rinses in PBST and 3 20-minute washes in PBST. The wing discs were mounted onto slides in 80% glycerol. The samples were analyzed using the Olympus FV1000 confocal microscope.

PCR

A three-step PCR was used, with a total 35 cycles. Initial denaturation was for 1 minute at 95°C, followed by an annealing step <2 minutes. The annealing temperature for the primer pairs are as follows: EcoRI + XbaI (58.2°C) used in cloning and sequencing; 446-426 + 426-446

(57°C) used for sequencing only. Finally, the extension lasted 1 minute at 72°C, with a final extension of 10 minutes at 72°C. For all diagnostic PCRs Taq polymerase (New England BioLabs) was used and for high fidelity applications the Q5 DNA polymerase was used (New England BioLabs).

RT-PCR

The primers used for RT-PCR analysis are listed in table 4.6. The primers (with the exception of RPA1) were designed to span exon-exon junctions. The PCR employed TAQ polymerase and included a total 35 cycles. Initial denaturation was 1 minute at 95°C, followed by an annealing step for 2 minutes (specific annealing temperatures are in table 4.6). Finally, the extension lasted 1 minute at 72°C with a final extension of 10 minutes at 72°C.

Table 4.6. List of all primers used for RT-PCR analysis.

Name	Sequence 5`-3`	Notes	Pair annealing temp
Tra-fem-2	TGGAACCCAGCATCGAGATTCC	Tra female specific (Tm = 71.3)	60°C
Tra-fem-qRT-rev	GAATCTTGTGCTCCTTCTTTCTG	Tra female specific (Tm = 68.9)	
Tra-male-qRT-fw	TGGAACCCAGCATCGAGTGTC	Tra male specific (Tm = 69.4)	60°C
Tra-male-qRT-rev	GCAGTTGAGAGTCCTATTGTCCT	Tra male specific (Tm = 63.2)	
RT-RPA1-REV	GTGAACGGATGGGTGCTACACAA	PCR Control (Tm = 60)	59°C
RT-RPA1-FOR	GACATGGGCTTCGCTCTCTT	PCR Control (Tm = 60)	
SxlRD Frw	CAACAAGTCGAGTGGTGGGC	Sxl female specific (Tm = 68)	59°C
SxlRD Rev	GCAACATGTGCCACCTGC	Sxl female specific (Tm = 66.8)	

Phenotype scoring for maternal effect of *cort*

Phenotype scoring was done on the male progeny from female *cort* homozygous mutant crosses crossed to male with either *Sxl* duplication, *sisA* duplication or *sisB* duplication; and female *cort* heterozygous mutants crossed to males with either *Sxl* duplication, *sisA* duplication or *sisB* duplication. To capture genital images, male flies were flash frozen with liquid nitrogen and stored at -80°C until all samples were collected. They were then thawed out and mounted for

the scanning electron microscopy. Pictures were taken using the Quanta 200 FEG (FEI) environmental SEM, on the low vacuum mode.

These crosses were carried out at 22 °C. The parental generation was transferred to fresh vials after 2 days for a total of 3 transfers, after which the parents were disposed off. The F1 progeny from these crosses were captured daily to prevent them from mating and laying eggs. Collection from a vial was stopped 9 days from when the first progeny eclosed. The F1 were sorted according to their genotypes and the males had their genitalia and analia examined under a light microscope. An individual was deemed to be abnormal if they were missing genital features (e.g. the male claspers), or if their genitals were oriented differently or were mangled. An individual was deemed highly abnormal if they displayed a complete or near complete lack of any genitalia and analia.

Statistical analysis

For the epistasis experiments: the average sex comb score was calculated for each condition, for both males and females. The graph in the figure represents averages from 3 separate trails; these averages were subjected to a one-tailed T-Test and one-way ANOVA with significance threshold set at 0.05 and 0.001 (respectively).

For the western blot experiments: A one-tailed T-Test was performed on the corrected band values obtained from 3 separate protein extractions and blots, with the significance threshold set at 0.05.

For maternal effect of *cort*: all adult progeny were counted, with the number of males from experimental cross represented as a percentage of number of males emerging from the control cross. For *Sxl* duplication percentage males from 4 separate trails is represented by the figure. Data analysis performed using a T-Test on these percentages from 4 separate trails, with a significance threshold of 0.05. Same procedure was used for sisB analysis, but data was collected from 3 separate trails.

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SUPPLEMENTRY FIGURES

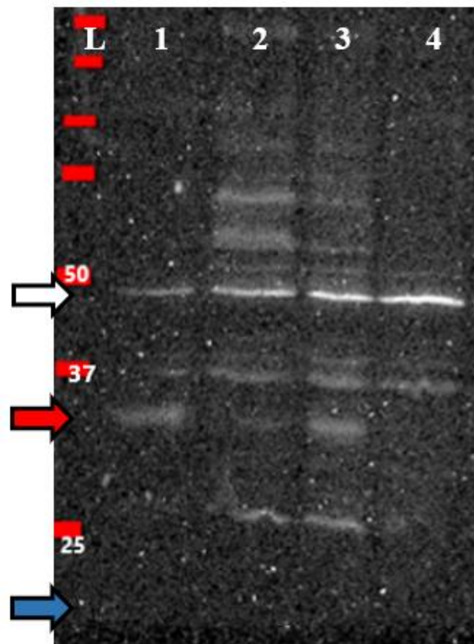

Figure S1. Probing for the Flag tag in samples prepared from female 3rd instar larvae resulting from crosses. Red arrow points towards the 35 kDa area on the blot. The white arrow points out the ~47 kDa protein used for loading correction. Blue arrow points out the location used to obtain the background noise value. **(Lane 1)** *HS-flag-tra^{f}* alone. **(Lane 2)** Coexpression of *UASp-HA-cort^{wt}(9)* and *HS-flag-tra^{f}*, the intensity of the Flag-Tra^{f} band goes down significantly. **(Lane 3)** expression of *HS-Flag-tra^{f}* with the *da-Gal4* to check for any possible effect of *da-Gal4* on HS-Flag-Tra^{f} levels. Flag-Tra^{f} protein band is visible, with no visible reduction. **(Lane 4)** female *yw* wildtype control, shows no detectable band at 35 kDa.

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