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Doublesex target genes in the red flour beetle, *Tribolium castaneum*

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Sex determination cascade in insects terminates with the production of sex-specific protein, Doublesex (Dsx). We identified the *dsx* homolog (*Tcdsx*) in *Tribolium castaneum*. The pre-mRNA of *Tcdsx* is sex-specifically spliced into three female (*Tcdsxf1*, *Tcdsxf2* and *Tcdsxf3*) and one male-specific (*Tcdsxm*) isoforms. *Cis-regulatory* elements potentially involved in sex-specific splicing of the *Tcdsx* pre-mRNA were identified in the female-specific exon and the adjoining intronic sequences. All the three female-specific TcDsx proteins share common OD1 and OD2 domains and differ in their C-terminal sequences. Knockdown of *Tcdsx* resulted in a reduction in the oocyte development, egg production and hatching of eggs laid. Several genes, including those coding for Vitellogenins and Vitellogenin receptors were identified as targets of TcDsx. RNAi experiments showed an isoform-specific targeting of identified target genes by TcDsx as knockdown in the expression of *Tcdsx* isoforms individually or in combinations resulted in differential effects on the expression of target genes.

Surprising diversity is shown by the organisms in their sex determination mechanisms[1,2]. Insects are no exception; different strategies are utilized by insects belonging to different orders to determine their sex during the early embryonic development[3–6]. The diversity in sex-determination mechanism lies in the presence of different upstream signals which ultimately work through the DM (*Doublesex-Mab3*) domain containing transcription factors[3,7]. The DM domain containing transcription factors are the best characterized sexual differentiation proteins[7,8] that are conserved among insects (*doublesex- dsx*)[9], worms (*male abnormal3- mab3*)[10] and vertebrates (*doublesex and mab3 related transcription* factor- *dmrt*)[11]. The *dsx* is the bottom most gene of the sex determination cascade in *Drosophila melanogaster*[12,13]. The pre-mRNA of *dsx* is sex-specifically spliced to produce one female- and one male-specific isoforms in turn generating one female (DsxF) and one male (DsxM) specific Dsx proteins, respectively. Sex-specific Dsx proteins share common DNA binding (DM or OD1) domain[14] but differ within their oligomerization domain (OD2)[15]. Due to this difference, sex-specific Dsx proteins have antagonistic effects on the regulation of their target genes involved in various aspects of sex differentiation[13,16]. Since the discovery of *dsx* in *D. melanogaster*, its homologues have been identified in several insect species belonging to orders Diptera[17–21], Hymenoptera[22,23] and Lepidoptera[24–26]. In most of the insect species studied, the pre-mRNA of *dsx* was found to be sex-specifically spliced to produce one female- and one male-specific RNAs. However, the *dsx* pre-mRNAs of *Musca domestica*[27] and *Apis mellifera*[22] are spliced to produce more than two splice variants. On the basis of open reading frame (ORF), one male- and one female-specific Dsx proteins are predicted. In *Aedes aegypti*[17], *Bombyx mori*[24,28] and other wild silkmoths[25] more than one *dsxf* transcripts have been identified which ultimately may generate more than one female-specific Dsx proteins. RNAi mediated knockdown studies showed the requirement of both the DsxF proteins in the female sexual differentiation of silkmoths[25]. Several indirect targets of Dsx have been predicted in *D. melanogaster*[29–31]. Recently, several direct targets of Dsx have been predicted, in *D. melanogaster*, based on the Dsx binding sites present in the promoter and intergenic region of these genes[32]. Very few genes have been functionally demonstrated to be the direct Dsx targets, among them *vitellogenin* (*vg*) is the best example[16,33]. The regulation in the expression of genes coding for *Vg*, Pheromone binding protein and *hexamerin* by Dsx has been shown in the lepidopteran insects[25,34,35]. Recent studies showed that *bric-a-bric* (*bab*)[36], *fad2*[37], and *wingless* (*wg*)[38] genes are also regulated by Dsx in *D. melanogaster*.

In spite of the fact that the insect order Coleoptera contains one fourth of all species described and includes many major pests of crop plants[39], nothing is known about the *dsx* and its targets in this group of insects. We identified and characterized the *dsx* homologue (*Tcdsx*) in coleopteran model insect, the red flour beetle, *Tribolium castaneum*. The pre-mRNA of *Tcdsx* is sex-specifically spliced to produce three female (*Tcdsxf1*, *Tcdsxf2* and *Tcdsxf3*) and one male-specific (*Tcdsxm*) isoforms. All the three female-specific *Tcdsx* isoforms are generated as a result of alternative splicing within the female-specific exon (exon3). Interestingly, putative

cis-regulatory elements were found in the female-specific *Tcdsx* exon and the adjoining intron sequences suggesting their possible involvement in the sex-specific splicing of *Tcdsx* pre-mRNA. We found several TcDsx target genes in *T. castaneum* by comparing the expression of previously identified female-specific genes in the control and *Tcdsx* RNAi insects. Knockdown in the expression of *Tcdsx* gene in an isoform-specific manner resulted in differential expression of identified target genes suggesting an isoform-specific regulation of target genes. The data included here confirm the evolutionary conserved role of *dsx* in insect sexual differentiation.

Results

Identification and characterization of *T. castaneum doublesex*. In order to identify *dsx* homolog (*Tcdsx*) in *T. castaneum*, blast (tblastn) searches were performed in the Beetlebase (http://beetlebase.org/) and in the NCBI (http://www.ncbi.nlm.nih.gov/) using known Dsx protein sequences as a query. A single sequence (ORF) of 969bp in length and annotated as "*Tribolium castaneum* similar to BmDSX-F (LOC660453)" was identified. Forward and reverse primers were designed based on this sequence (LOC660453). Three fragments were amplified when cDNA made using RNA isolated from females was used as a template in RT-PCR. Whereas, only one fragment was amplified when cDNA made using RNA isolated from male was used as a template (data not shown). Sequencing and analysis of sequence of these fragments showed that 'LOC660453' is a male-specific isoform (*Tcdsxm*) of *Tcdsx*. The three female isoforms identified are named as *Tcdsxf1*, *Tcdsxf2* and *Tcdsxf3*. UTR sequences were obtained by sequencing 3′ and 5′ RACE PCR products which were confirmed by aligning them with the *Tcdsx* genomic sequences (AAJJ01001880.1 and AAJJ01000060.1). Further, full length *Tcdsx* splice variants were amplified by RT-PCR using sex-specific cDNA and primers specific to the ends of *Tcdsx* (Fig. 1A); three female-specific amplicons of 2264bp (*Tcdsxf1*), 2428bp (*Tcdsxf2*) and 2186bp (*Tcdsxf3*) and one male-specific amplicon of 1788bp (*Tcdsxm*) were obtained (Fig. 1B). Because, the difference between *Tcdsxf1* and *Tcdsxf3* is only 78bp, they migrate closely in the gel

Figure 1 | (A) Schematic representation of isoforms of *Tcdsx* pre-mRNA, showing the primer positions and regions used for preparation of dsRNA. Boxes show exons and lines show introns. The sizes (bp) of different exons are shown within the exons. Blue colored regions represent the ORF whereas the orange colored regions represent UTRs. Four different splice variants of *Tcdsx* pre-mRNA, three female- (*Tcdsxf1*, *Tcdsxf2* and *Tcdsxf3*) and one male-specific (*Tcdsxm*), are produced. A(n) show the polyadenylation site. Vertical lines represent start and stop codon sites. Vertical arrows show the domains of the TcDsx proteins whereas horizontal arrows show primer positions. Green horizontal lines show regions corresponding to dsRNA; ds-*dsxC* (common) = 354 bp, ds-*dsxf1+f2* = 78 bp and ds-*dsxf2*= 120 bp. Primers F1 and R1 were used to amplify full length *Tcdsx* transcripts and primer qRTCF was used with either qRT*f1*R, qRT*f2*R or qRT*f3*R in qPCR for the quantification of specific *Tcdsxf* transcripts. The sequences of all the primers mentioned here are given in supplementary Table 2. (B) Gel picture showing three bands (*Tcdsxf1*, *Tcdsxf2 and Tcdsxf3*) in females and one band in male (*Tcdsxm*) as a result of RT-PCR using sex-specific cDNA as template and primers (F1 and R1) specific to ends of *Tcdsx* (Fig. 1A). M represents DNA size marker. C) Gel picture showing three bands (*Tcdsxf1*, *Tcdsxf2 and Tcdsxf3*) in females and one band in male (*Tcdsxm*) as a result of RT-PCR using sex-specific cDNA as template and internal primers (F2 and R2) spanning the alternatively spliced region of *Tcdsx* (Fig. 1A). Same primers (F2 and R2) were used for analyzing the splicing status of *Tcdsx* in previous paper[55]. M represents DNA size marker.

(Fig. 1B). The PCR fragments were cloned and sequenced, and analysis of sequences confirmed the presence of two products in *Tcdsxf1/f3* bands. Further, to show the presence of three female-specific *Tcdsx* splice forms, RT-PCR was performed using internal primers and sex-specific cDNAs; three female- and one male-specific amplicons were amplified (Fig. 1C). The conceptual translation of ORFs of these sex-specific isoforms showed the presence of DM and OD domains confirming the existence of three female- and one male-specific *Tcdsx* isoforms. Full length cDNA sequences and the deduced amino acid (aa) sequences of *Tcdsx* have been submitted to GenBank (accession no. for *Tcdsxm*, *Tcdsxf1*, *Tcdsxf2* and *Tcdsxf3* are JQ857098, JQ857099, JQ857100 and JQ857101, respectively).

Genomic organization of *Tcdsx* and proteins encoded by *Tcdsx* isoforms. The *Tcdsx* transcript sequences span 8503bp region in AAJJ01001880.1 and 23138bp region in AAJJ01000060.1 genomic contigs (Fig. 1A). Exon-intron boundaries were assigned based on the alignment of *Tcdsx* cDNA sequences with the corresponding genomic DNA sequences (AAJJ01001880.1 and AAJJ01000060.1). *Tcdsx* gene harbors 6 exons and 5 introns; except for exon 3 which is female-specific, all others are common to both male and female (Fig. 1A). Generation of three female-specific transcripts is due to alternative splicing within female-specific exon (exon 3). The deduced amino acid sequences of three TcDsxF proteins differ with each other only at their C-terminal ends. First two exons, common to all the female- and the male-specific transcripts, code for 197aa common N-terminus region of TcDsx proteins. Due to the alternative splicing within the third exon 55, 30 and 29 aa are added to TcDsxF1, TcDsxF2 and TcDsxF3 respectively (Fig. 2A and Fig. S1). Multiple sequence alignment of sex-specific TcDsx proteins with the Dsx proteins from other insects (*D. melanogaster*, *A. gambiae* and *B. mori*) showed a high degree of sequence conservation in the N-terminal region containing DM/OD1 domain and in the common region containing the OD2 domain (Fig. 2A). Interestingly, amino acids residues (C, H, H, C, C and R) within the DM/OD1 domain shown to be essential for DNA binding activity in *D. melanogaster*[14,41] were found to be 100% conserved in TcDsx proteins (Fig. 2A). Consistent with this, very high sequence conservation was also found in the C-terminal regions of the DsxF proteins (Fig. 2B). Very little similarity was observed in the male-specific regions of Dsx proteins (Fig. 2C). Unlike Dsx proteins identified from other insects, the male-specific TcDsx protein (TcDsxm) contains two OD2 domains (OD2a and OD2b). Whereas, female-specific TcDsx proteins contain only one OD domain (OD2a). TcDsxM and TcDsxF are identical at their N-terminus until the OD2a domain of TcDsxM (OD2a is truncated in males but complete in females) but differ at their C-terminal region; OD2b is specific to TcDsxM (Supplementary Fig. 1S). Supplementary Table 1 summarizes the length of mRNA, protein and number of exons present in different *Tcdsx* isoforms.

Putative splicing regulatory elements in *Tcdsx*. In-silco analysis of splice sites (donor/acceptor sequences) of *Tcdsx* showed the presence of GT at all the splice donor sites and AG at the intron junctions between exons 2, 4 and 5 (Table 1). The splice acceptor site preceding the exon 3 also contains AG but the additional introns generated in*Tcdsxf1* and *Tcdsxf3* (see Fig. 1) contain CT at their splice acceptor sites. The splice acceptor site in the intron preceding exon 6 contains TC rather than AG (Table 1). We did not find any repeats of putative dipteran Tra/Tra2 binding sequence in the genomic sequence of *Tcdsx*; the female-specific exon of *dsx* in dipteran insects contain imperfect repeat of 13nt (RE-Repeat Element)[17–19,42]. Interestingly, three repeats (A/CGAAGAAA/G) matching the putative *dsx* RE sequence of *A. mellifera* and *Nasonia vitripennis*[43] were found exclusively in the intron downstream to the female-specific exon (exon3); these repeats are clustered within 552bp region. A Purine Rich Stretch (PRE- GAAGAAGTAGAGAA) was also identified exclusively in the 3′ region of exon 3 (Supplementary Fig. 2S). Clustering of the splicing regulatory sequences in the female-specific exon and the adjoining intron sequences suggests their possible involvement in the sex-specific splicing of the *Tcdsx* pre-mRNA.

RNAi studies on *Tcdsx*. *T. castaneum* is an excellent coleopteran model insect owing to the fact that RNAi woks efficiently in this beetle[44]. dsRNA 1) targeting common region of *Tcdsx* which could silence all the female- and male-specific transcripts, 2) targeting two female-specific transcripts, *Tcdsxf1* and *Tcdsxf2* together and 3) targeting only one female-specific transcript, *Tcdsxf2* (Figure 1A), were synthesized and injected into newly eclosed sex-separated pupae of *T. castaneum*. Total RNA isolated from 5 day-old adult's eclosed from injected pupae was used to quantify *Tcdsx* mRNA levels. Injections of dsRNA caused an efficient knockdown in the expression of targeted *Tcdsx* isoforms (Supplementary Fig. 3S). The phenotypes observed in *Tcdsx* knockdown insects are described below.

Effect of Tcdsx *RNAi on oocyte development*. In *T. castaneum*, oocyte development takes place during the first few days post adult emergence (PAE)[45,46]. Ovaries were dissected from 5th day PAE females, eclosed from pupae injected with *Tcdsx* or *malE* dsRNA, stained and observed under a microscope. When compared to the oocytes in the control beetles injected with *malE* dsRNA (Fig. 3A), there was a significant reduction in the size of oocytes in the females injected with dsRNA targeting all the three *Tcdsx* transcripts together or *Tcdsxf1* plus *Tcdsxf2* or *Tcdsxf2* alone (Fig. 3B, 3C and 3D, respectively). The primary oocytes in control insects matured to stages 6-7 [see *Parthasarathy et al*[46] for description of oocyte stages] whereas, the primary oocytes in *Tcdsx* dsRNA injected insects were arrested at stages 1–4. Injection of *Tcdsx* dsRNA (targeting common region) in males affected testis development; the lobes in the testis of *Tcdsx* RNAi insects are smaller than that of control insects injected with *malE* dsRNA (Fig. 3E and 3F). These data suggest the requirement of TcDsx proteins for the development of oocytes and testis.

Effect of Tcdsx *RNAi on egg production and embryogenesis*. We also determined the effect of *Tcdsx* knockdown on egg production and hatching of eggs laid, by crossing female and male adult beetles eclosed from pupa injected either with *Tcdsx* or *malE* dsRNA. Crosses were done in four different combinations; *Tcdsx* RNAi females with *Tcdsx* RNAi males, *Tcdsx* RNAi females with *malE* RNAi males, *malE* RNAi females with *Tcdsx* RNAi males and *malE* RNAi females with *malE* RNAi males. No eggs were produced by the *Tcdsx* RNAi females mated with *Tcdsx* RNAi or control (*malE* RNAi) males in all the *Tcdsx* dsRNA injected beetles (Table 2). Females developed from uninjected pupae or pupae injected with *malE* dsRNA and mated with males developed from uninjected pupae or pupae injected with *malE* dsRNA laid the same number of eggs. Very few eggs were laid by the females mated with *Tcdsx* RNAi males but these eggs hatched and developed into larvae (Table 2).

Effect of Tcdsx *RNAi on* vg *mRNA levels*. The gene coding for Vitellogenin (Vg) has been characterized as a target for Dsx protein in *D. melanogaster*[47]. Previous studies have shown that the basal levels of *vg* gene expression is up regulated by the female form of Dsx protein (DsxF) and down regulated by the male form of Dsx protein (DsxM)[16,33,47,48]. In *T. castaneum*, two genes [*vg1* (Glean-13602) and *vg2* (Glean-10839)] coding for Vg have been identified and both the genes show identical patterns of expression[49]. Both *vg1* and *vg2* mRNA levels were quantified in *Tcdsx* knockdown and control insects. dsRNA injections, targeting common region of *Tcdsx*, lead to a significant ($p<0.05$) reduction in both *vg1* and *vg2* mRNA levels in females (Fig. 4A). Knockdown of *Tcdsxf1* and

A Common region

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                                                                                        DM/OD1
TcDsx    M-S---------SDSQDFDSKMDVN--ASSTSAS---PRTPPNCARCRNHRLKIALKGHKRYCKYRTCKCEKCRLTTERQRVMAMQTA 73
DmDsx    MVSEENWNSDTMSDSDMIDSKNDVCGGASSSSGSSISPRTPPNCARCRNHGLKITLKGHKRYCKFRYCTCEKCRLTADRQRVMALQTA 88
AgDsx    MVSQDRW-AEAMSDSG-YDSRTDGNG-ASSSCNNSLNPRTPPNCARCRNHGLKIGLKGHKRYCKYRTCHCEKCCLTAERQRVMALQTA 85
Bmdsx    MVSMGSWKRRVPDDCE---ERSEPG--ASSSG----VPRAPPNCARCRNHRLKIELKGHKRYCKYQHCTCEKCRLTADRQRVMAKQTA 79
         **           *             ***       ** ********** *** *********    * *******  ****** ***

TcDsx    LRRAQAQDEA------------------------------------------------------------------------------ 83
DmDsx    LRRAQAQDEQRALHMHEVPPANPAATTLLSHHHHVAAPAHVHAHHVHAHHAHGGHHSHHGHVLHHQQAAAAAAAAPSAPASHLGGSST 176
AgDsx    LRRAQTQDEQRALNEGEVPPEPV----------------------------------------------------------------- 108
Bmdsx    IRRAQAQDEARARALELGIQPP------------------------------------------------------------------ 101
          **** ***

TcDsx    ------------------------------------------------------MLRS------GSAVDPAIMQVPLKSPSPIHAIE 110
DmDsx    AASSIHGHAHAHHVHMAAAAAASVAQHQHQSHPHSHHHHHQNHHQHPHQQPATQTALSPPHSDHGGSVGPATSSSGGGAPSSSNAAAR 264
AgDsx    ------------------------------------------ANIHIPKLSELKDLKHNMIHNSQTRSFDCDSSTGSMASAPGTSSVP 154
Bmdsx    --------------------------------------------------------------------GMELDRPVPPVVKAPRSPMIP 122

TcDsx    RSLDCDSS------------------ASSQCSNPPPAIRKMTPVP--------------AVPSSTS-VNIGTIAQSTD---------- 155
DmDsx    ATSSNGSSGGGGGGGGGSSGGGAGGGRSSGTSVITSADHHMTTVPTPAQSLEGSCDSSSPSPSSTSGAAILPISVSVNRKNGANVPLG 352
AgDsx    LTIHRRSP------------------GVPHHVA---EPQHLGATH---------------SCVSPEPVNLLP--------------- 190
mdsx     PSAPRSLG-----------------SASCDSVPGSPGVSPYAPPP--------------SVPPPPTMPPLIPTPQPPVP--------- 170

                 OD2
TcDsx    ---LLEDCQKLLERFKYPWEMMPLMYAILKDARADLEEASRRIDE 197
DmDsx    QDVFLDYCQKLLEKFRYPWELMPLMYVILKDADANIEEASRRIEE 397
AgDsx    DDELVKRAQWLLEKLGYPWEMMPLMYVILKSADGDVQKAHQRIDE 235
Bmdsx    SETLVENCHRLLEKFHYSWEMMPLVLVIMNYARSDLDEASRKIYE 215
                    ***   * ** ***   *    *      *   * *
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B Female specific Region

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AgDsx     GQAVVNEYSRLHNLNMFDGVELRNTTR---Q--------SG--------------- 30
DmDsx     GQYVVNEYSRQHNLNIYDGGELRNTTR---Q--------CG--------------- 30
BmDsx     GKMIVDEYARKHNLNVFDGLELRNSTR---QKMLEINNISGVLSSSMKLFCE---- 49
TcDsxF2   GKRVVNEYSRLHNLNMYDGVELRNSTR---Q--------YG--------------- 30
TcDsxF1   GKRVVNEYSRLHNLNMYDGVELRNSTHNKNQDRRSFSSNPSFKSVNLQFKCNVLI 55
TcDsxF3   -----------------D---------NKNQDRRSFSSNPSFKSVNLQFKCNVLI 29
          *    * ** * ****  ** **** *     *
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C Male specific Region

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AgDsx    GKRTIKTYEALVKSSLDPNSDRLTEDDEDENISVTRTNSTIRSRSSSLSRSRSCSRQAETPRADDRALNLDTKSKPSTSSSSGTGCDR 88
DmDsx    -----------ARVEINRTVAQIYYNYYTPMALVNGAPMYLTYPS------------------------------------------ 34
TcDsx    ---------GRDTEILLDFCQRLKDKFQLSWKMISLVDVILKYAK------------------------------------------ 36
BmDsx    ---------------------------------------------------------------------------------------

AgDsx    DDGDCITFDDSASVVRATHASRSATRMSRGRSRSQTKRYSQTVESTNAPSRSPGPDEEPSVYKSLAEAASKMARSFIPAREPEDLHTT 176
DmDsx    --------------IEQGRYGAHFTHLPLTQICPPTPEPLALSRSPSSPSGPSAVHNQKPSRPGSSNG------TVHSAASPTMVTTM 102
TcDsx    ----------------DQDEAWRQIDEAFLEIRALAAVEAARYTYHHIPYSGLYPNAATAIYPPVYLP------SMSMYHPATLLGSV 102
BmDsx    ------------GYWMMHQWRLQQYSLCYGALELSARKDVAALCCLR--DTCWRPRSRRVWCPSS---------------------- 51
                                             :

AgDsx    THKSPEREDNPSQPYEAYLESVRRSKKSFPHKDAEGVTESAEDCYDKEKEHRIPYSLPKSTFDRLDLLKKPNGLPFPMYKYNELEANN 264
DmDsx    ATTSSTP---------------------------------------------------------------------------------- 109
TcDsx    PTSTSPS---------------------------------------------------------------------------------- 109
BmDsx    ----------------------------------------------------------------------------------------- 51

AgDsx    FPLPLLLPGLEAVNRTLYTAHFPTHLLPSSLYPPVSSESTTAPIFHTHFLGYQPQMQLPHVEPFYRKEQQQQQLQQTLAEPKEQTTSS 352
DmDsx    ---------------------------------------------------------TLSRRQRS------------------- 117
TcDsx    ----------------------------------------------------------------------------------------
BmDsx    ----------------------------------------------------------------------------------------

AgDsx    SPSNNRLTPPKGTFFYASAVENSLTAHQAS--IATIH 387
DmDsx    -RSATPTTPPPPPPAHSSSNGAYHHGHHLVSSTAAT- 152
TcDsx    ------HSPPIVPRAIRPSSRA--------------- 125
BmDsx    -------------------------------------
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Figure 2 | Deduced amino acid (aa) sequences of Dsx proteins from *T. castaneum* (TcDsx), *D. melanogaster* (DmDsx), *A. gambiae* (AgDsx) and *B. mori* (BmDsx) were aligned. The Dsx sequences are divided into (A) region common to DsxM and DsxF proteins (B) Female-specific region which is known to be conserved and C) Male-specific region. The DNA binding domain (DM/OD1) is shown in blue color whereas oligomerization domain (OD2) is shown in orange color. Arrows indicate the conserved amino acid residues (in red) shown to be essential for DNA binding activity of Dsx in *D. melanogaster*. The three TcDsxF proteins differ from each other at their C-terminus (B). Additional OD2 domain in TcDsxM is shown in orange color (C). Stars (*) represent 100% identity of aa among all proteins.

Table 1 | Exon-intron junction sequence of *Tcdsx* gene

Exon No.	Isoform	Exon size (bp)	Splice donor	Intron size (bp)	Splice acceptor
1	Common	577	CGATCG/gtgagtcgtcctatgc	?	—
2	Common	141	ATGAAG/gttagtgtgacgattg	167	gtattcattgtttcag/CTCAAA
3F	*Tcdsxf1*	78	CAACGC/gtcagtatggatgatt	144	aatacacttaatttag/GAAAGC
		416	TATAAG/gtaagtcttgcagctg		gtaatgcatgtcccct/TTTTTC
	Tcdsxf2	638	TATAAG/gtaagtcttgcagctg	No Intron	aatacacttaatttag/GAAAGC
	Tcdsxf3	416	TATAAG/gtaagtcttgcagctg	222	gtaatgcatgtcccct/TTTTTC
4	Common	141	ATGAAG/gtaaggcatctagctg	1945	tgtgtttgtttggcag/GTCGTG
5	Common	242	ATAAAG/gtaagggtacttgctt	970	ttgtacttatttccag/CATTCC
6	Common	773		8450	tttatgtcattgtttc/AGACGT
—	Tcdsxm	No male specific Exon	ATGAAG/gttagtgtgacgattg	2750	tgtgtttgtttggcag/GTCGTG

The exon-intron junction sequence corresponding to different *Tcdsx* transcripts are shown. Upper case letters represent exonic sequence whereas lowercase letters represent intronic sequences. ? = the size of intron 1 could not be determined because of sequence gap.

Tcdsxf2 together, also lead to a significant reduction in both *vg1* and *vg2* mRNA levels in females. No significant reduction in both *vg1* and *vg2* mRNA levels were observed in females injected with *Tcdsxf2* dsRNA, as compared to the control females injected with *malE* dsRNA (Fig. 4A). Both *vg1* and *vg2* mRNA levels in male *Tcdsx* RNAi insects were significantly higher ($p<0.05$) as compared to their levels in control males injected with *malE* dsRNA (Fig. 4B).

Identification of TcDsx target genes. Microarray studies using RNA isolated from sex-separated adults of *T. castaneum* insects identified several genes expressed in a female-specific manner (data not shown). We selected 30 female-specific genes based on their minimum of 5-fold expression difference between female and male and tested the possibility of these genes as targets of TcDsx protein. cDNA made from RNA isolated from *Tcdsx* RNAi (knockdown of a single or a combination of *Tcdsx* isoforms) and control beetles and primers (supplementary Table 2) specific to the selected female-specific genes were used in the qRT-PCR analysis to quantify mRNA levels. Out of the 30 genes tested, 12 genes (including two *vg* and two *vg* receptor genes) showed a reduction in their mRNA levels in *Tcdsx* RNAi females compared to that in control females suggesting that these genes may be regulated by TcDsx (Fig. 4A and 4B).

Differences in the expression of these genes in beetles injected with dsRNA targeting different isoforms of *Tcdsx* were also observed. On this basis, these genes were categorized into two groups; I) genes, whose expression is down regulated when the dsRNAs targeting the common region or the region specific to both, *Tcdsxf1* and *Tcdsxf2* transcripts were injected (Fig. 4A) and II) genes, whose expression is down regulated when dsRNA targeting the common region of *Tcdsx* is injected (Fig. 4C). These data suggest the possibility of regulation of different genes by TcDsxF proteins in an isoform-specific manner. Interestingly, there is no difference in the expression of any of these target genes in the females injected with dsRNA targeting only *Tcdsxf2* isoform (Fig. 4A and 5C) suggesting a possible functional redundancy of TcDsxF2 withTcDsxF1 and TcDsxF3. We did not find any significant difference in the down regulation of these genes when dsRNA targeting the common region was injected compared to when dsRNAs specific to *Tcdsxf1* and *Tcdsxf2* transcripts were injected (data not shown). Injection of dsRNA targeting common region of *dsx* into males caused a significant increase in mRNA levels of both *vg* genes and G07776 (Fig. 4C) suggesting that TcDsxM suppresses expression of these three genes in males. The expression of other nine genes tested was not affected in *Tcdsx* RNAi male beetles.

Direct Dsx target genes in *D. melanogaster* are known to harbor a 13bp palindromic sequence to which Dsx binds[15,32]. Computational analysis of the whole genome sequence of different insect species besides dipterans suggest an evolutionary conservation and enrichment of Dsx-binding sequence in their genome[32]. In order to search for the Dsx binding site(s) and their location in the identified TcDsx targets we scanned the genomic region corresponding to each gene (the region of genomic DNA containing gene + 3 kb upstream to their ATG site) for the presence of sequence(s) similar to Dsx binding

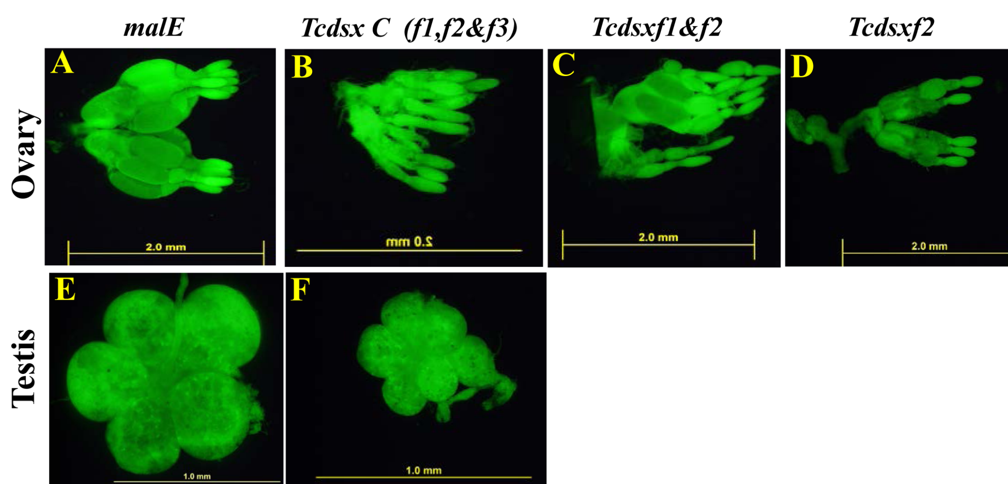

Figure 3 | *Tcdsx* RNAi affects development of ovary and testis. Young (0 day) sex-separated pupae were injected with dsRNA targeting all isoforms of *TcdsxC* (*f1*+*f2*+*f3*), *Tcdsxf1*+ *Tcdsxf2* (*f1*+*f2*), *Tcdsxf2* alone or *malE*. Ovaries or testis were dissected on 5th day PAE, stained with acridine orange and photographed at 4X (ovary) or 10X magnification (testis) using fluorescence microscope.

Table 2 | Effect on fecundity after knockdown of *Tcdsx* transcripts

dsRNA	RNAi female X RNAi male	Control female X RNAi male	RNAi female X Control male
TcdsxCommon	0	4.2±1.5	0
Tcdsxf1+f2	0	67±2.6	0
Tcdsxf2	0	68.5±2.4	0

The values shown are Mean±S.D. (n=6). Crosses between control female and control males produced 67±3 eggs per pair.

sites identified in *D. melanogaster*. Interestingly, we noticed the presence of 13 bp consensus sequence(s) in eight (G03183, G08596- Vg receptor, G04042- Vg receptor, G15076- Proteoglycan 4, G13602-Vg1, G10839- Vg2, G14653- Lysosomal acid lipase, and G07776) of these genes (Fig. 5A and 5B) suggesting that these genes may be the direct targets of TcDsx proteins. These Dsx binding sequences are composed of two half-sites around a central (A/T) base pair, the core (7 bp) region of which was found to be palindromic and highly conserved as reported for Dsx binding sites in *D. melanogaster*[32]. These putative TcDsx binding sites are located in the promoter or intergenic regions (except in G13602 where one of the putative Dsx binding sequences is also found in exonic region). Table 3 summarizes the identified *Tcdsx* target genes, corresponding genomic contig, tissue of expression and their putative functions.

Discussion

One of the final steps of sex determination cascade in insects is the production of female- or male-specific Dsx proteins[4,9,50]. Production of one female- and one male-specific *dsx* isoforms (in turn one female- and one male-specific Dsx protein) is considered to be the trade mark feature of *dsx* in the insect species studied so far. The existence of one female- and one male-specific Dsx proteins was unchallenged until the discovery of multiple female-specific *dsx* pre-mRNAs, differing in their ORFs, in silkmoths[25,28] and yellow fever mosquito[17]. Identification of multiple isoforms of *Tcdsx* and the results from the RNAi-mediated knockdown of these splice variants in different combinations supports the requirement of more than one DsxF proteins in the female sexual differentiation. All the three female-specific *Tcdsx* transcripts are expressed during larval (final instar larvae), pupal and adult stages (data not shown). The expression of four genes (G03183; G08596,Vg receptor; G04042,Vg receptor and G15076,Proteoglycan 4) was reduced in females injected with dsRNA targeting the common region of *Tcdsx* transcripts but unaffected by the injection of dsRNA targeting *Tcdsxf1* plus *Tcdsxf2* transcripts (Fig. 4C). There could be two possibilities for the observed difference in both the cases; 1) the knockdown efficiency of *Tcdsxf1* and *Tcdsxf2* transcripts are different in both the cases since the dsRNA targeting different regions were synthesized and they varied in their lengths (Fig. 1A) and/or 2) these genes are regulated by TcDsxF3 protein alone. Quantitative analysis of the levels of *Tcdsxf1* and *Tcdsxf2* transcripts in the insects injected with dsRNA targeting either common region or *Tcdsxf1* and *Tcdsxf2* transcripts specifically showed almost equal amount of reduction of these transcripts in both the cases (Supplementary Fig. 3S). Hence, we conclude that TcDsxF3 protein is the sole regulator of these genes. Other group of genes showed highly reduced expression in females injected with dsRNA targeting common region or *Tcdsxf1* plus *Tcdsxf2*; the expression of these genes was unaffected by injection of dsRNA specific to *Tcdsxf2* alone. At present, it is difficult to conclude whether the expression of these genes is regulated by TcDsxF1 or TcDsxF3 or by both these proteins. Either we did not identify any TcDsxF2 target gene or function of TcDsxF2 is redundant with other TcDsxF isoforms. Presence of underdeveloped oocytes in the females injected with dsRNA targeting *Tcdsxf2* alone suggests a role for *Tcdsxf2* in oocyte development and hence most likely TcDsxF2 target genes were not identified in our analysis. Further, the presence of putative TcDsx binding sequences in some of the TcDsx target genes (Fig. 5A and 5B) suggest them to be the direct targets of TcDsx. Other genes (G07775, G05384, G07186 and G06658) in which no conserved Dsx binding sites were identified are most likely indirect targets of TcDsx as Dsx in *D. melanogaster* is known to regulate many transcription factors which in turn regulate the expression of genes[51].

The female-specific exon 4 of *D. melanogaster dsx* contains a stretch of purine nucleotides which makes the preceding 3′ splice site a weak splice acceptor and therefore overlooked by the spliceosomal machinery in males, resulting in the skipping of exon 4[52]. In females, Transformer (Tra), a female-specific protein along with Tra2 (a non sex-specific protein) and with other splicing regulatory proteins binds to the *dsx* repeat elements (*dsx*RE) making the 3′ splice site stronger resulting in the incorporation of exon 4 in female-specific *dsx* isoform[52–54]. We recently characterized the homolog of *tra* (*Tctra*) in *T. castaneum* and showed its requirement for the female-specific splicing of *Tcdsx*[55]. The *Tctra* pre-mRNA, in males, splices in a manner which retains male-specific exons with several in-frame stop codons in it, resulting in the production of non-functional protein[55]. Several *cis*-regulatory sequences were found to be located exclusively in the female-specific exon and its adjoining intron of *Tcdsx*. Together with the observation of skipping of exon 3 in males and presence of a stretch (14 bp) of purine rich sequence (GAAGAAGTAGAGAA), in the 3′ end of female-specific exon suggest this as putative Purine Rich Element (PRE) of *Tcdsx* which most likely makes the 3′ splice site preceding exon 3 as a weak splicing acceptor site. Besides, clustering of putative *cis*-regulatory elements (putative Tra/Tra2 binding sequences and Tra2-ISS elements) was found in the female-specific exon (exon 3) and the adjacent intron sequences. Identification of these putative *cis*-regulatory elements together with the previous data[55] suggest the interaction of TcTra/Tra2 protein complex and other splicing regulatory proteins with the *cis*-regulatory elements present in *Tcdsx*, in females, and regulate female-specific splicing (retention of exon3) of *Tcdsx* pre-mRNA. However, the mechanisms of regulation of alternative splicing within the exon 3 and generation of three female-specific *Tcdsx* isoforms remains unknown. Presence of weak splice acceptor site (CT) within exon 3 might be playing a role in alternative splicing of female-specific exons. Similar kind of alternative splicing within the female-specific exons of *dsx* leads to the generation of two female-specific splice variants with different ORFs in lepidopteran insects[25,28]. Studies on differential expression of target genes in beetles injected with dsRNA targeting one or more *Tcdsx* isoforms and effect of knockdown in the expression of specific *Tcdsx* isoforms on oocyte development provided some initial clues on isoform-specific functions of *Tcdsx*. However, further studies are needed to clarify isoform-specific functions of *dsx*.

Methods

***Tribolium castaneum* strain, RNA isolation and RT-PCR.** The GA-1 strain of the red flour beetle, *Tribolium castaneum* was used in all the studies. Organic whole wheat flour containing 10% yeast was used for rearing the beetles. Pupa and adults were sexed based on the presence of sex-specific structures. RNA was isolated using Trizol method (Invitrogen Corporation, USA). DNAse treated total RNA was denatured at 75°C for 5 min and immediately chilled on ice. First strand cDNA was synthesized with MMLV reverse transcriptase (Invitrogen, USA) using 17-mers polyT primer. Initial denaturation at 94°C for 2 min, 32 cycles of 94°C for 30 s, 60°C for 30 s, 72°C

Figure 4 | Relative expressions of female-specific genes in *Tcdsx* or *malE* dsRNA injected beetles. Young (0 day) sex-separated pupae were injected with dsRNA targeting all isoforms of *TcdsxC* (*f1+f2+f3*), *Tcdsxf1+ Tcdsxf2* (*f1+f2*), *Tcdsxf2* alone or *malE*. Total RNA was isolated on 5th day PAE and the mRNA levels of selected genes were quantified by qRT-PCR. Mean+S.D (n=3) relative mRNA levels are shown. Asterisks show treatments that are significantly different from control ($p<0.05$). (A) TcDsx target genes that are down regulated in females injected with dsRNA targeting either common region of *Tcdsx* or the region specific to *Tcdsxf1* and *Tcdsxf2* (*f1+f2*) together. (B) Female-specific genes that significantly increased in *Tcdsx* RNAi males. (C) TcDsx target genes that decrease only when dsRNA targeting the common region of *Tcdsx* was injected into females. The expressions of these genes are unaffected in females injected with dsRNA targeting *Tcdsxf1* and *Tcdsxf2* together. All these genes are unaffected by the injection of dsRNA targeting *Tcdsxf2* alone.

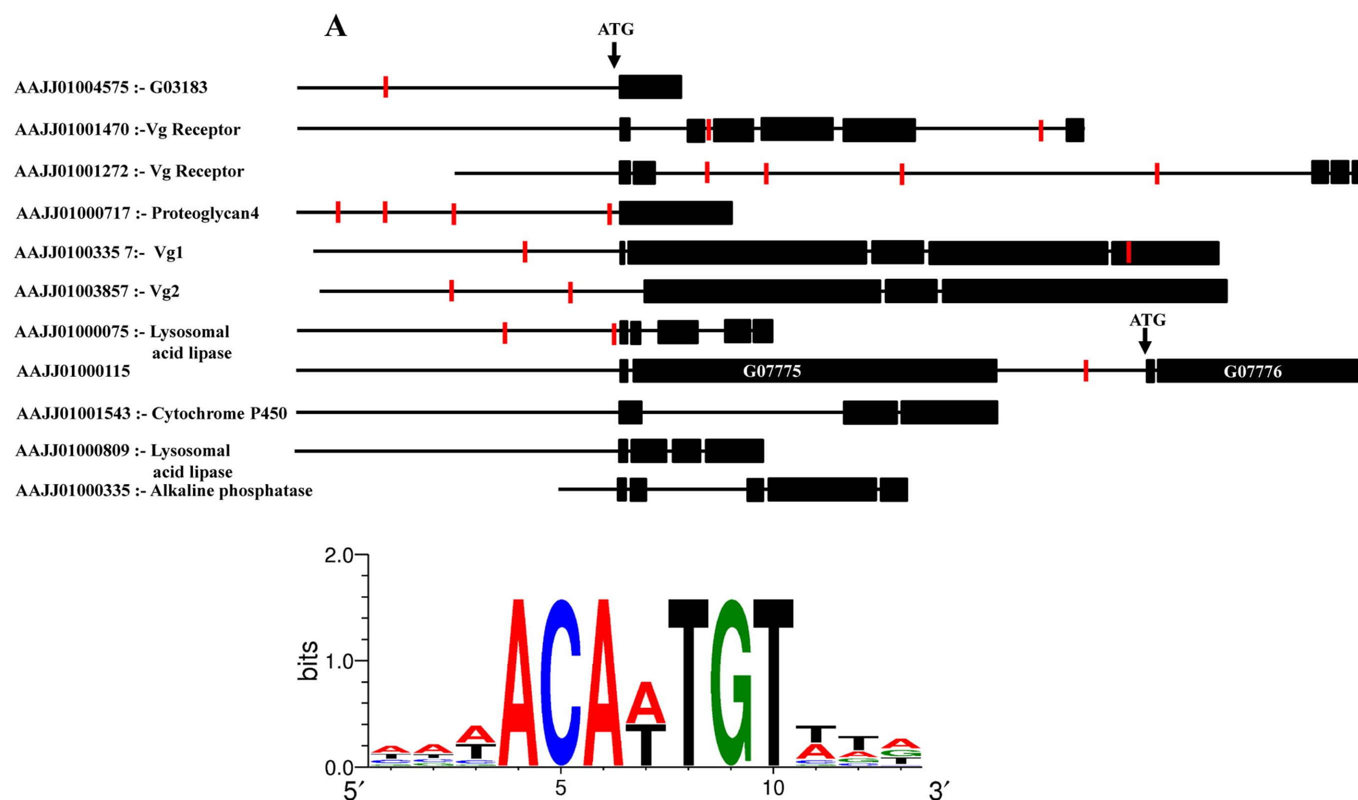

Figure 5 | (A) Schematic representation of genes showing the presence of putative Dsx binding site sequences in the putative promoter and intergenic region of eight (G03183, G08596- Vg receptor, G04042- Vg receptor, G15076- Proteoglycan 4, G13602- Vg1, G10839- Vg2, G14653- Lysosomal acid lipase, and G07776) of the TcDsx target genes. (B) Weblogo, showing the nucleotide identity of the putative TcDsx binding site sequences in the identified TcDsx target genes. The height of each stack indicates the consensus sequences at that position (in bits) whereas the height of nucleotides represents the relative frequency of each nucleotide at that position.

for 2 min and the final extension at 72°C for 10 min were used to perform PCR reactions.

3′ and 5′ RACE PCR. 3′ and 5′ RACE reactions were performed using SMARTer RACE cDNA Amplification Kit (Clontech, USA) according to manufacturer's instruction, using adapter- and gene-specific primers. The primary and nested gene-specific primers used for 3′ RACE PCR of *Tcdsx* are 3′GSP1_dsx (5′-GATCAAGACGAGGCGTGGAGGCAGAT-3) and 3′GSP2_dsx (5′-CGCCGTCGAAGCAGCAAGGTACAC-3′) The primary and nested gene-specific primers used for 5′ RACE PCR are 5′GSP1_dsx (5′-CTCGGTGGTCAGCCGGCACTTCT-3′) and 5′GSP2_dsx (5′-CTTGTGGCCTTTGAGGGCGATCTTGA-3′).

Drop down PCR reaction with conditions, initial denaturation at 94°C for 2 min, 5 cycles of 94°C for 30 s, 72°C for 2 min, 5 cycles of 94°C for 30 s, 70°C for 2 min, 25 cycles of 94°C for 30 s, 68°C for 2 min, 72°C for 2 min and a final extension at 72°C for 10 min were performed on Biorad master cycler. Amplicons of different sizes were gel-eluted, sequenced and the 5′ and 3′ *Tcdsx* sequences were selected based on the overlapping regions of known sequence of *Tcdsx*. Sequences were further confirmed by RT-PCR using primers specific to the known sequence and the sequences obtained through RACE experiments.

Sequence analysis. Exons and introns, of the *Tcdsx* were identified by aligning sequences of RT-PCR products with their corresponding genomic DNA sequences obtained from the Beetlebase (http://beetlebase.org/) and the NCBI (http://www.ncbi.nlm.nih.gov/). Exon-intron boundaries were confirmed by aligning the sequences through Spidey program (http://www.ncbi.nlm.nih.gov/spidey/).

Double stranded RNA (dsRNA) synthesis and injections. Primers specific to different isoforms of *Tcdsx* containing the T7 promoter sequence at their 5′ ends and cDNA were used to amplify different fragments of *Tcdsx*. Purified PCR products were used as templates to synthesize dsRNA using MEGAscript T7 kit (Ambion, Austin, TX). A fragment from *Escherichia coli malE* gene was used to prepare control dsRNA. dsRNA injections were performed into newly eclosed (0 day) pupae. The insects were kept on ice for 8–10 minutes prior to injections. dsRNAs (≈500–600 ng per insect)

Table 3 | List of female-specific genes identified as TcDsx targets

Group	Glean Number	Corresponding Contig	Expression in tissue	Annotation	Putative Dsx binding sites
I	G03183	AAJJ01004575	Ovary	Hypothetical protein	Yes
	G08596	AAJJ01001470	Ovary	Vg receptor	Yes
	G04042	AAJJ01001272	Ovary	Vg receptor	Yes
	G15076	AAJJ01000717	Ovary	Similar to proteoglycan 4	Yes
II	G13602	AAJJ01003357	Fat body	Vg1	Yes
	G10839	AAJJ01003857	Fat body	Vg2	Yes
	G14653	AAJJ01000075	Fat body/Ovary	Similar to lysosomal acid lipase	Yes
	G07775	AAJJ01000115	Fat body/Ovary	Hypothetical protein	No
	G07776	AAJJ01000115	Fat body/Ovary	Hypothetical protein	Yes
	G05384	AAJJ01001543	Fat body/Ovary	Cytochrome p450	No
	G07186	AAJJ01000809	Fat body	Similar to lysosomal acid lipase	No
	G06658	AAJJ01000335	Fat body/Ovary	Alkaline phosphatase	No

were injected on the ventral side of the pupae using an aspirator tube assembly (Sigma-Aldrich) fitted with 3.5′′ glass capillary tube (Drummond) pulled by a needle puller (Model P-2000, Sutter Instrument Co.). Injected pupae were allowed to recover for 8 hr at room temperature (~22°C) and then were transferred to standard containers containing food. Knockdown efficiencies of gene expression in the RNAi insects were calculated as the ratio of gene expression between 5 day-old (adult) beetles eclosed from pupae injected either with target dsRNA or *malE* dsRNA.

Quantitative real time PCR. Quantitative PCR was performed using the SYBR Green kit (Roche, USA) according to the manufacturer's instructions. RNA isolation and RT-PCR was done as mentioned above. Three independent biological replicates were included for each treatment. Expression of *T. castaneum rp49* gene was used as an endogenous control to normalize the expression data and the gene expression levels were analyzed by $2-\Delta\Delta Ct$ method[40].

Imaging and documentation. The ovaries and testes were dissected from the staged insects and stained with acridine orange and the images were taken using Olympus 1×71 Inverted Research Microscope fitted with reflected fluorescence system. Acridine orange was excited using 502 nm laser. "Megna Fire software" (version 1.5) was used to control the microscope, image acquisition and exportation of TIFF files. Figures of all micrographs were assembled using Adobe Photoshop element 9.

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Author contributions

Both authors planned and conducted experiments and wrote manuscript.

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