Characterisation of the M-locus and functional analysis of the male-determining gene in the housefly
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Chapter 4
Expression pattern and functional analysis of
*Mdmd* in *Musca domestica*
4.1 Abstract

In *Musca domestica*, a positive auto-regulatory feedback loop ensuring female-specific splicing of *Mdtra* mRNA in the zygote eventually leads to female development. The male-determining gene(s) act to interrupt this loop, resulting in male-specific splicing of *Mdtra* mRNA and leads to male development. The *M*-locus that contains the male-determining gene(s) has a complex organisation with repetitive sequences of which only one appears to contain an intact open reading frame (ORF). This ORF was assumed to be part of the male-determining gene *Mdmd*. The *Mdmd* ORF sequences on autosomes II, III and V and on the Y-chromosome are highly similar, but homologous sequences cannot be found on autosome I, which apparently has a different male-determining gene(s). Knockdown of *Mdmd* by RNAi silencing confirmed that *Mdmd* is necessary for male development and knockout of *Mdmd* by CRISPR-Cas9 resulted in complete feminisation. To identify the expression pattern of *Mdmd*, *in situ* hybridisation (ISH) with DIG-labelled *Mdmd* RNA probe was performed. It unveiled the ubiquitous expression of *Mdmd* mRNA throughout embryonic development, indicating that *Mdmd* already acts at a very early embryonic stage to stop the *Mdtra* loop and it is probably continuously needed to maintain male development of the zygote. This corroborates the transient effects observed in earlier RNA interference experiments. To study whether *Mdmd* activity is solely sufficient for male development, functional *Mdmd* capped, polyadenylated RNA was injected in early blastoderm stage embryos that are a mixed set of male and female embryos. If *Mdmd* is sufficient for male development, transient expression of *Mdmd* in genotypic female embryos should lead to all male offspring. However, no sex-reversed flies were obtained, although an insignificant bias towards more males was observed in injected offspring. These results either indicate that expression of *Mdmd* alone is not sufficient to turn genotypic females into males or alternatively, it is caused by an insufficient translation of *Mdmd* mRNA.
4.2 Introduction

In the common housefly, *Musca domestica*, *Md-transformer (Mdtra)* mRNA and *Mdtra2* mRNA is maternally provided to kick-start a positive autoregulatory feed-back loop of female-specific splicing of *Mdtra* mRNA in the zygote that leads to female development in the absence of a dominant male-determining gene(s) (Burghardt et al., 2005; Bopp, 2010, Hediger et al., 2010). MdTRA protein leads to female-specific splicing of *Mdtra* mRNA with the assistance of other essential co-factors such as MdTRA2 protein (Burghardt et al., 2005; Hediger et al., 2010). MdTRA protein and its co-factor MdTRA2 protein subsequently splice the mRNA of *M. domestica doublesex, Mddsx*, into the female variant, which leads to female development (Burghardt et al., 2005; Hediger et al., 2010). The male-determining gene(s) act to interrupt the auto-regulatory loop, resulting in male-specific splicing of *Mdtra* mRNA and a non-functional MdTRA truncated protein (Hediger et al., 2010). Hence, in the presence of a male-determining gene(s), *Mddsx* mRNA is spliced into its male-specific isoform, leading to male development. The *M*-locus that contains the male-determining gene(s) is typically located on the Y-chromosome, but can also be present on any autosome or even the X-chromosome (Wagoner, 1969; Inoue and Hiroyoshi, 1982; Denholm et al., 1983; Inoue et al., 1986).

Recently, based upon differential expression analysis, the male-determining gene, *Mdmd (Musca domestica male determiner)* was identified in *M. domestica* (Sharma et al., 2017). A BLAST search of the *Mdmd* sequence against the published female genome (Scott et al., 2014) showed that *Mdmd* has a high sequence similarity with the splicing regulatory gene *CWC22/ncm (nucampholin)* (Sharma et al., 2017). Structural analysis of the *M*-locus revealed multiple copies of partial homologous sequences (reported in chapter 2). Upon analysis of these repetitive sequences, only one appeared to contain an intact open reading frame (ORF). The *Mdmd* ORF sequences on autosomes II, III and V and on the Y-chromosome are highly similar to each other, but they cannot be found on autosome I, which apparently has a different male-determining gene(s) (reported in chapter 3). This ORF was assumed to be the coding sequence of *Mdmd*, the functional male-determining gene.

*Mdmd* is only present in the male genome and zygotic *Mdmd* was first detected within 2-3 hrs after egg laying (cellularised blastoderm stage), which corresponds with the timing of the appearance of *Mdtra* male-specifically spliced transcripts (Hediger et al., 2010; Sharma et al., 2017). Sharma et al. shows that *Mdmd* is continuously expressed in adult male flies, indicating that male development potentially needs continuous activation of *Mdmd*. Besides,
transcripts of Mdtra and its co-factor Mdtra2 are abundantly present in embryos (D. Bopp, unpublished results; Burghardt et al., 2005). In this chapter, I present the temporal and spatial distribution Mdmd mRNA in developing embryos with *in situ* hybridisation (ISH). *In situ* hybridisation is a powerful tool to localise specific nucleic acid sequences in tissues, providing insights into the regulation and organisation of target genes. *In situ* hybridisation is first performed on a 8-10 hrs old mixed set of male and female embryos because a large amount of Mdmd transcripts is expressed during this stage (Sharma et al., 2017). Next, to observe the expression pattern of Mdmd in developing embryos, *in situ* hybridisation is also performed on a 0-24 hrs old mixed set of male and female embryos. Localisation of Mdmd expression in developing embryos by *in situ* hybridisation will help to clarify the expression patterns of Mdmd and shed light on its regulation of Mdtra in the *M. domestica* sex determination pathway.

Knockdown of Mdmd by RNAi silencing confirmed that Mdmd is necessary for male development and knockout of Mdmd by CRISPR-Cas9 resulted in complete feminisation (Sharma et al., 2017). This shows that Mdmd plays a crucial role in male development. However, these experiments did not show whether Mdmd is solely sufficient for male determination. Based on the fact that the Mdmd ORF is conserved throughout *M. domestica* strains, Mdmd is expected to be solely sufficient to perform the male-determining function in *M. domestica* and MdmdV will then act similarly as MdmdIII in the MIII strain. In this chapter, I aim to introduce functional MdmdV into early blastoderm stage embryos that are a mixed set of male and female embryos from the MIII strain by injecting capped, polyadenylated RNA after *in vitro* synthesis. Transient expression of MdmdV with capped, polyadenylated RNA in embryos is a good way to study the function of MdmdV as capped RNA of MdmdV mimics the function of the male-determining gene found *in vivo*. If transient expression of MdmdV in the early blastoderm female embryos turns genotypic females into males, it means that MdmdV is sufficient for male development in *M. domestica*.

### 4.3 Materials and Methods

#### 4.3.1 *Musca domestica* strains and culturing

The 3-6 MIII strain is used for *in situ* hybridisation and transient expression of MdmdV. In this strain, M is located on autosome III. Females have genotypes X/X; pw bwb w/pw bwb w and males X/X; pw+ MIII bwb+ w/pw + bwb w. pw stands for pointed wings, bwb for brown body and w for white eyes, all being recessive visible markers on autosome III. Females have brown body, white eyes and pointed wings. Males are heterozygous for M and they have black body, white
eyes and normal wings. Strains were reared at 25°C as described previously (Schmidt et al., 1997).

4.3.2 Embryo collection and fixation for in situ hybridisation

In situ hybridisation was performed on a 8-10 hrs and a 0-24 hrs old mixed set of male and female embryos. 8-10 hrs old embryos were selected because a large amount of Mdmd is expressed during this stage (Sharma et al., 2017). Embryos of the MIII strain were collected and washed in Milli-Q water. The embryos’ chorion was removed by treating them with 50% chlorix diluted in Milli-Q water for 2-3 min. Embryos were fixed for 25 min in 1.5 mL Eppendorf tubes with a 1:1 mixture of 4% paraformaldehyde (PFA) and n-Heptane after vigorously shaking for 30 sec to allow the fix to penetrate the vitelline membrane. After fixation, the lower phase (fixation solution) was removed and an equal amount of methanol was added. Tubes were then immediately shaken for 15 sec to remove the vitelline membrane and to sink embryos to the bottom. Embryos were washed twice in methanol and subsequently stored at -20°C prior to in situ hybridisation.

4.3.3 Preparation of RNA probe for Mdmd

In situ probes were synthesised from a PCR template with the Mdmd-specific primers MdmdF and MdmdR1, which are located in ORM#1. ORM#1 is one of the orphan contigs of Mdmd that was identified among the top male-specifically expressed sequences that were absent in the female genome (Sharma et al., 2017). The ORM#1 region is relatively dissimilar from Md-ncm to avoid interference with Md-ncm during hybridisation. The reverse primer MdmdR1 used to synthesise the antisense probe contains a T7 promoter sequence. The forward primer MdmdF used to synthesise the sense probe contains an SP6 promoter sequence (Fig. 4.1). PCR conditions were: 1 µL plasmid DNA that contains MdmdV cDNA (100 ng/µL), 0.5 µL 10 µM MdmdF, 0.5 µL 10 µM MdmdR1, 2 µL 2.5 mM dNTP, 5 µL 5×Phusion HF Buffer and 0.5 µL Phusion enzyme in a total volume of 25 µL. PCR was performed by denaturation at 94°C for 2 min, then 30 cycles denaturation at 94°C for 30 sec, annealing at 70°C for 30 sec and extension at 72°C for 1 min, and lastly extension at 72°C for 10 min. PCR products were analysed on a 1% agarose/EtBr gel. The target fragment was purified with the NucleoSpin® Gel and PCR clean-up kit from Macherey-Nagel (Düren, Germany).

In situ probes were synthesised through in vitro transcription. The transcription reaction was set up with the following concentrations: 6.5 µL mixture containing 300-500 ng DNA template + DEPC water, 1 µL Dig labelling mix, 1 µL
10×transcription buffer, 0.5 µL RNasin (40 U/µL), 1 µL T7/SP6 RNA polymerase in a total volume of 10 µL. Transcription was performed at 37°C for 2 hrs, after which the reaction mixture was diluted with 40 µL RNA free water. 5 µL of diluted probes were tested in the gel with 25 ng of template DNA in the neighbouring lane as a control. After testing, 5 µL 3 M NaAc and 125 µL 100% EtOH were added to the probe, followed by precipitation at -20°C overnight. The following day, the mixture was centrifuged at 16,000 g at 4°C for 30 min and the supernatant was removed. 1 mL 70% EtOH was added to the tube, which was then centrifuged at 16,000 g at 4°C for 10 min. The supernatant was removed and the pellet air-dried. The pellet was re-suspended in 50 µl resuspension buffer and stored at -20°C.

**Figure 4.1:** *Mdmd*-specific primers used for *in situ* hybridisation probes: *MdmF* and *MdmR1* are both located in the ORM#1 region. The blue sequence is the SP6 promoter sequence and the red sequence the T7 promoter sequence. ORM#1, ORM#2, ORM#3 and ORM#6 are four orphan contigs of *Mdmd*. MIF4G and MA3 are two conserved domains (Sharma et al., 2017).

### 4.3.4 *In situ* hybridisation of *Mdmd* in embryos

Fixed embryos stored at -20°C in methanol were gradually rehydrated by washing in 50% methanol/PBT (1×), then twice in 100% 1×PBT. Embryos were post-fixed in 1:1 mixture of 1×PBT and 4% paraformaldehyde (PFA) in 1.5 mL Eppendorf tubes for 20 min. They were then washed in 1×PBT three times for 20 min each. After that, embryos were shortly washed in a 1:1 mixture of 1×PBT and Hybe A solution before being shortly washed in the Hybe A solution three times. Embryos were then incubated at 65°C in Hybe A solution for 1 hr. 30 µL Hybe A solution was mixed with 1 ng *in situ* probe, followed by incubating in 95°C for 2 min to degenerate the probes. The mixture was cooled on ice for 1 min and pre-warmed to 65°C. Embryos were subsequently incubated overnight at 65°C in a mixture of probe + Hybe A solution. The following day, they were washed three times for 20 min at 65°C in the Hybe A solution and shortly washed in 1:1 mixture of 1×PBT and Hybe A solution, followed by three times washing in 1×PBT for 20 min each. Embryos were then incubated in 1×PBS with anti-Dig antibody (1:2000) for 1 hr. This was followed by three times washing
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for 20 min in 1×PBT. Hybridisation signals were visualised with an alkaline phosphatase-based detection system. Embryos were washed three times in NBT buffer for 5 min each prior to NBT/BCIP staining (4.5 µL NBT and 3.5 µL BCIP in 1 mL NBT buffer) in the dark. The staining was stopped by several washes in 1×PBT. After that, embryos were incubated in 1×PBT containing 1:1000 dilution of 4', 6-diamidino-2-phenylindole (DAPI) for 30 min in the dark. Embryos were washed with PBT several times in the dark before being stored at 4°C in 100% glycerol.

4.3.5 Multiplex single-embryonic PCR

To verify the sex of embryos, multiplex single-embryonic PCR was performed according to the modified protocols of Horn and Wimmer (2003). Male-specific primer combinations F2 and cDNA_R_MII_MV or F1 and R4 that only amplified target fragments of Mdmd in males were used to distinguish the male and female embryos. Stained and unstained embryos from in situ hybridisation were independently homogenized in 50 µL squishing buffer plus 0.5 µL proteinase K (20 mg/mL). The homogenate was incubated at 55 °C for 1 hr and at 95°C for 6-7 min to inactive proteinase K. PCR was performed with the following concentrations and conditions: 5 µL homogenate, 0.5 µL 10 µM forward primer, 0.5 µL 10 µM reverse primer, 2 µL 2.5 mM dNTP, 5 µL 5×Phusion HF Buffer and 0.5 µL Phusion enzyme in a total volume of 25 µL; samples were denaturated at 94°C for 2 min, followed by 35 cycles of 94°C denaturation for 30 sec, annealing at 59°C for 30 sec and 72 °C for 4 min, and finally extension at 72°C for 10 min. PCR products were analysed on a 1% agarose/EtBr gel. Primers F2 and cDNA_R_MII_MV or primers F1 and R4 were used to identify the presence of Mdmd that only exists in males. Primers MdnmcF1 and MdnmcR1 were used to amplify the Md-ncm that is present in both males and females.

4.3.6 Microscopy

In situ stained embryos were imaged on a Carl Zeiss (Oberkochen, Germany) Axioplan2e, 10×Plan Neofluar objective (0.3 Numerical aperture), 1.4 MP monochrome camera Retiga (Q-imaging, Surrey, Canada), with ImagePro software (version 6.3, Media Cybernetics inc., Silver Spring, United States). Differential interference contrast (DIC) was used to image the in situ stainings of the embryos. DAPI staining was recorded in 10 focal planes with a filter set from Zeiss (Set 49). Extended depth of field (EDF) imaging was performed with generate composite best-focus image (output options), large edges (focus regions), normalize illumination (focus analysis options) combined with top down stack order. Embryonic in situ stainings were visualised under a Leica
M205 FA fluorescence stereomicroscope (Wetzlar, Germany) and documented with camera MicroPublisher 5.0 RTV (Q-imaging, Surrey, Canada) and ImagePro software (version 7.01, Media Cybernetics Inc., Silver Spring, United States).

4.3.7 Preparation of template DNA for synthesising functional MdmDV mRNA

A linearised plasmid DNA that contains an RNA polymerase promoter site was used as template for in vitro transcription of MdmDV. In chapter 3, I described the cloning of the MdmDV cDNA to the pCR®II vector that has a T7 promoter sequence upstream and an SP6 promoter sequence downstream of the insertion site (Fig. 4.2). MdmDV cDNA contains 90bp un-translated sequences upstream and 17 bp un-translated sequences downstream of the coding sequence. A unique restriction site for the enzyme SpeI (A|CTAGT) that is located downstream of the MdmDV insertion is chosen to linearise the plasmid DNA with the following concentrations and conditions: 1 µg plasmid DNA, 0.5 µL SpeI from Promega (10 u/µL; Wisconsin, United States), 0.2 µL acetylated BSA (10 µg/µl), 2 µL restricted enzyme 10×Buffer B in a total volume of 20 µL, followed by incubating at 37°C for 3 hrs. The digestions were checked on a 1% agarose/EtBr gel and the target fragments were isolated by Wizard® SV Gel and PCR clean-up system from Promega (Wisconsin, United States).

Figure 4.2: Map of pCR®II+MdmDV construct. There are a T7 promoter upstream and an SP6 promoter downstream of the MdmDV insertion site. The MdmDV cDNA contains 90bp un-translated sequences upstream and 17 bp un-translated sequences downstream of the coding sequence. SpeI is a unique restriction site downstream of the MdmDV insertion.

4.3.8 Capped in vitro transcription and Poly(A) tailing of MdmDV

The linearised plasmid was used to synthesise capped and polyadenylated MdmDV mRNA in vitro by using the mMESSAGE mMACHINE® Kit and Poly(A)
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Tailing Kit from Ambion (Massachusetts, United States). The reaction was set up with 5 µL of linearised plasmid DNA template (171.3 ng/µL), 10 µL of T7 2×NTP/ARCA (15 mM ATP, 15 mM CTP, 15 mM UTP, 3 mM GTP, 12 mM ARCA), 2 µL 10×T7 Reaction Buffer, 2 µL of T7 enzyme mix and 1 µL of 30 mM GTP in a total volume of 20 µL, followed by incubating at 37°C for 3 hrs. After the in vitro transcription, poly(A) tailing was performed by adding 36 µL of nuclease free-water, 20 µL of 5×E-PAP Buffer, 10 µL of 25 mM MnCl₂, 10 µL 10 mM ATP solution into 20 µL mMessage mMachine reaction. 2.5 µL of the reaction mixture was used as a negative control. After that, 4 µL of E-PAP was added to the remaining reaction mixture. The mixture was incubated for 1 hr at 37°C and placed on ice afterwards. 2.5 µL was removed and checked on a denaturing agarose gel together with 2.5 µL of the reaction mixture without the poly(A) tail. In the final step, RNA was recovered by the MEGAclear™ Kit from Ambion (Massachusetts, United States).

2% denaturing agarose gel were prepared with the following concentration and conditions: 2 g agarose (Standard Agarose, Type LE) from BioConcept (Salem, United States) was dissolved in 72 mL DEPC water and was cooled to 60°C. 18 mL 37% formaldehyde solution (12.3 M) and 10 mL 10×MOPS running buffer were added to the dissolved agarose. Before loading the gel, 2.5 µL of RNA sample or an appropriate RNA marker was premixed with 7.5 µL Formaldehyde Load Dye that contains 10 µg/mL Ethidium bromide. Mixtures were heated and denatured at 75 °C for 10 min and then 10 µL of samples/marker was loaded onto the 2% denaturing agarose gel. The concentration of recovered RNA was checked with NanoDrop from Thermo Fisher Scientific (Massachusetts, United States).

4.3.9 Embryo microinjection for germline transformation

Dechorionation and microinjection of embryos were performed as described previously (Hediger et al., 2001). 0-1 hr old early blastoderm mixed set of male and female embryos from the MIII strain were collected for microinjection. Embryos were dechorionated by washing them with a sodium hypochlorite solution for 2-3 min, followed by several washes with tap water to remove the remaining sodium hypochlorite, and finally rinsed twice with Ringer’s solution. Embryos were aligned along the edge of cover slips under the microscope, desiccated for 3 min in a chamber filled with dry Silica gel and submerged with 10S:3S Voltalef oil of VWR (with 4:1 mix ratio; Pennsylvania, United States). Cover slips with embryos were kept on a glass plate on ice to slow down embryo development. Embryos were injected in the posterior end with a sharpened glass needle filled with capped and polyadenylated MdmdV RNA. After microinjection,
Voltalef oil was removed and cover slips were put on an agar plate and incubated overnight in a desiccator filled with oxygen at 18 °C. Living larvae from injected embryos were raised on porcine faeces as it is difficult to keep small numbers of larvae on standard medium (Hediger et al., 2001). Adult flies were screened after hatching to search for masculinised individuals.

4.4 Results

4.4.1 Embryonic Mdmd expression detected by in situ hybridisation

Sense and antisense probes for in situ hybridisation were successfully synthesised using SP6 polymerase and T7 polymerase, respectively, (Fig. 4.3). In-situ hybridisation with the anti-sense RNA probe revealed the presence of large amounts of Mdmd mRNA in 8-10 hrs old embryos (Fig. 4.4). Some embryos were stained and some were not. The detected Mdmd mRNA in the embryos are male-specific, because no Mdmd is detected in female embryos (Fig. 4.5). The ubiquitous expression of Mdmd started at early embryonic stage (blastoderm stage) and continued until late embryogenesis (dorsal closure stage) (Fig. 4.6).

![Figure 4.3: Probe synthesis of Mdmd. Lane1 is PCR product amplified by MdmdF and MdmdR1 primer combination (DNA template). Lane 2 is antisense probe and lane 3 is sense probe. The DNA template is 660bp in length.](image-url)
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Figure 4.4: *Mdmd* is ubiquitously expressed in a subset of 8-10 hrs old embryos.

Figure 4.5: *Mdmd* is male specifically expressed in 8-10 hrs old embryos. A: Embryo stained by antisense probe and PCR confirmed that it is a male embryo. B: Embryo stained by antisense probe and PCR confirmed that it is a female embryo. C: Embryo stained by sense probe served as a negative control. D: Embryos stained by *apterous* (*ap*) served as a positive control. *ap* is expressed in embryonic head and in segmentally repeated pattern (arrows). Primers F2 and cDNA R_MIII_MV were used to amplify *Mdmd* that is only present in males. Primers MdnncmF1 and MdnncmR1 were used to amplify *Md-ncm* that is present in both males and females.
Figure 4.6: *Mdmd* is continuously expressed during embryo development. High levels of uniform staining are found from blastoderm stage (A) to dorsal closure stage (B). Embryos are staged by a counterstaining with DAPI.

*Mdmd* could not be detected by PCR in some of the stained embryos (Fig. 4.7). For example, embryos #M2, #M3 and #M4 do show staining, but *Mdmd* was not detected in embryo #M3 (Fig. 4.7, lanes 2-4). Lanes 5 and 6 contain unstained embryos (embryos #13 and #14) and there is no target fragment amplified as expected. The absence of *Mdmd* derived products could be due to polymorphism in the primer regions in different individuals. To test this hypothesis, PCR was performed on genomic DNA from adult flies with *Mdmd*-specific primers. *Mdmd* could be amplified from gDNA of all adult males, whereas no *Mdmd* was amplified from gDNA of adult females (Fig. 4.8), suggesting that no polymorphism affecting the PCR exists in this region. Therefore sequence polymorphism cannot explain why *Mdmd* could not be detected by PCR in some of the stained embryos. Another explanation is background staining, which is further discussed in the discussion section.

Figure 4.7: PCR amplification for *Mdmd* on stained and unstained embryos. Lane 1 contains male genomic DNA that served as positive control. Lanes 2-4 contain stained and lanes 5-6 contain unstained embryonic DNA. Lane 7 contains female genomic DNA that served as negative control. Primers F1 and R4 were used to amplify *Mdmd* that is only present in males and primers
MdncmF1 and MdncmR1 were used to amplify *Mdncm* that is present in both males and females.

![PCR amplification for Mdmd on genomic DNA of adult flies. Lanes 1-11 contain gDNA of adult males. Lanes 12-14 contain gDNA of adult females. Primers F1 and R4 were used to amplify *Mdmd* that is only present in males and primers MdncmF1 and MdncmR1 were used to amplify *Mdncm* that is present in both males and females.](image)

**4.4.2 Functional analysis of *Mdmd* by transient expression**

To study whether *Mdmd* is solely sufficient to turn genotypic females into males, functional *Mdmd* capped, polyadenylated RNA was injected in *M. domestica* early blastoderm stage embryos that are a mixed set of male and female embryos from the *M* III strain. Capped polyadenylated transcription of *Mdmd* was performed prior to microinjection and yielded a 3.5kb transcript (Fig. 4.9, lane 1). Next, poly(A) tailing of capped RNA was performed to increase the stability of mRNA (Fig. 4.9, lane 2). Capped, polyadenylated RNA was purified for microinjection (Fig. 4.9, lane 3).

![Capped and polyadenylated RNA synthesised with the mMESSAGE mMACHINE® Kit. Lane 1 contains untailed capped RNA, lane 2 tailed capped RNA and lane 3 purified tailed capped RNA by MEGAclean™ Kit from Ambion (Massachusetts, United States).](image)
Transient expression ofMdmd\textsuperscript{V}did not yield masculinised individuals among 1906 injected embryos. 63 males and 43 females survived to adulthood, among which all males had black bodies and normally shaped wings and all females had brown bodies with pointed wings (Table 4.1). None of the females showed external masculinisation. Groups that were injected with different concentrations ofMdmd\textsuperscript{V}capped, polyadenylated RNA did not have significantly different sex ratios ($\chi^2 = 0.0686, p = 0.7933, \text{d.f.} = 1$). However, a bias towards more males was observed in both groups, which was marginally insignificant ($\chi^2 = 3.8397, p = 0.05005, \text{d.f.} = 1$). As capped polyadenylated RNA expression could be transient, it may have only influenced gonads of developing embryos. Therefore, all 43 females were dissected but found to have ovaries, indicating that transient expression ofMdmd\textsuperscript{V}in the early blastoderm female embryos did not reverse genotypic females into males.

Table 4.1: Results of microinjection ofMdmd\textsuperscript{V}capped, polyadenylated RNA. 106 Flies out of 1906 injected embryos survived into the adult stage. Microinjection of capped, polyadenylatedMdmd\textsuperscript{V}RNA did not transform the genotypic females into males.

<table>
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<th>Concentration</th>
<th>Embryos</th>
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<th>Adults (\text{♀})</th>
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* Adults from embryos injected by 500 ng/\muL and 1 \mu g/ \muL capped polyadenylated RNA are counted together.

4.5 Discussion

In this study, I had two main objectives regarding the function ofMdmd in M. domestica. The first objective was to determine the expression pattern ofMdmd in developing embryos to better understand its regulation in sex determination. The second objective was to find out whether expression ofMdmd is solely sufficient to turn genotypic females into males.

High levels of uniform staining are found in developing embryos. In M. domestica, female sex is promoted by maternally inheritedMdtra, which needs to remain continuously active to maintain the female promoting function. However,
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The presence of Mdmd inhibits the Mdtra auto-regulatory loop, promoting male differentiation. Male-specific Mdtra transcripts first appeared 2-3 hrs after egg laying, indicating that Mdmd acts very early during development to stop the Mdtra loop (Hediger et al., 2010). *In situ* hybridisation revealed that Mdmd mRNA are present in early blastoderm stage embryos. In addition, a large amount of Mdmd mRNA was detected throughout embryo development, indicating that Mdmd starts to act very early during embryonic development to stop the Mdtra loop and embryos need continuous activation of Mdmd to maintain their male development. This corroborates the transient effects observed in earlier RNA interference experiments of Mdmd, which leads to development of male individuals with fully differentiated ovaries, indicating that Mdmd restored its activity at late embryonic stage and caused the incomplete feminisation (Sharma et al., 2017).

Following *in situ* hybridisation, some of the stained embryos failed to yield an Mdmd-specific product in PCR. PCR amplification with the same primer set on genomic DNA from adult flies successfully detected Mdmd in all males, whereas Mdmd could not be amplified in females. Therefore, it is unlikely that Mdmd was not amplified in some of the stained embryos due to polymorphism in the primer sequences in different individuals. Other factors, such as background staining, may explain the observed false-positives. Reabsorbing the antibodies, reusing previously utilized antibodies or stopping the antibody reaction as soon as the staining in male embryos appears, may help to reduce such background staining.

There are many successful studies of gene function by *in vitro* synthesised mRNA and microinjection of ectopic transcripts into embryos. For example, Krzywinska et al., (2016) injected Yob mRNA into Anopheles gambiae embryos to confirm its male-determining function. Transient expression of MdmdV with capped, polyadenylated RNA did not yield any masculinised flies in my study. One possible explanation is that a synthetic copy was not sufficient to transform genotypic females into males, which may indicate that additional genes along with MdmdV are required to induce male development. Hediger et al. (1998) reported that there are at least two male-determining factors (M) located on each arm of the Y-chromosome. They showed that the M on the short arm of the Y-chromosome provides almost full masculinising activity, whereas the M located on the long arm has a weak masculinising activity. However, due to lack of sequence information of M, it is not clear whether these two M copies are identical or represent two different genes that perform the male-determining function. At this moment, the results presented here combined with results from previous studies (Hediger et al., 1998) suggest that additional genes may be involved in male determination.
An alternative explanation is that *MdmdV* actually is sufficient for male determination, but this could not be adequately proven due to technical shortcomings. First, it is difficult to control the translational timing of mRNAs (Mimoto and Christian, 2011). In some cases, translated mRNA can persist for several days in embryos, but it may also persist for only a few hours. I cannot exclude that mRNA was translated immediately after microinjection and only persisted for some hours.

Second, the stability of capped RNA greatly depends on the poly (A) tail that increases the stability of the mRNA and the efficiency of its translation (Harland and Misher, 1988; Bernstein and Ross, 1989; Gallie, 1991). For example, in the developing *Xenopus* embryo, the half-life of capped RNA transcripts that have a long poly (A) tail of 200 nucleotides is 6-8 hrs, twice as long as capped RNA transcripts that lack a poly (A) tail (Harland and Misher, 1988). Poly(A) tails of 60-80 nucleotides do not have a reliable stabilisation effect on injected RNAs in embryos (Harland and Misher, 1988). I did not observe size differences in capped RNA before and after polyadenylation, but as the poly(A) tail is only approximately 150bp, the gel running time may not have been long enough to see such small difference. Alternatively, polyadenylation may also have failed to produce a poly(A) tail of adequate length. If so, transcripts may have been degraded in less than 8 hrs. If transcripts of *MdmdV* was degraded before completely halting the activity of the *Mdtra* feedback loop, the feedback loop may have re-established its own activity, thereby inducing female development.

Third, the complete sequences for 5′ and 3′ un-translated regions (UTRs) of *MdmdV* cDNA are still unknown. The 5′ and 3′ UTRs contain sequence elements that are essential for the post-transcriptional regulation (Barrett et al., 2012). The incomplete sequences in 5′ and 3′ UTRs of *MdmdV* cDNA could hinder the efficient translation of *MdmdV* mRNA. A fourth possible technical problem may be that *MdmdV* encodes a long 3.5kb transcript, which prevents efficient transcription of *MdmdV*, and generates truncated transcripts. Truncated *MdmdV* transcripts would not yield any functional Mdmd protein, resulting in a failure to halt the activity of the *Mdtra* feedback loop and consequently no masculinisation of genotypic females. Finally, the concentration of capped and polyadenylated *MdmdV* RNA might not have been adequate to disrupt the *Mdtra* auto-regulatory loop.

To overcome the technical problems in microinjection of *MdmdV* with capped, polyadenylated RNA and to answer the question whether expression of *Mdmd* is actually sufficient to turn genotypic females into males, two experiments need to be done in the near future. The major advantage of injecting capped,
polyadenylated RNA is that it can be used to study the function of genes in vivo without germline transformation, which is more laborious because one needs to select a suitable transposable element and construct a transgene. Thus, microinjection needs to be repeated with higher concentrations of capped, polyadenylated RNA. Cloning the intact cDNA of Mdmd is also required for efficient translation of Mdmd mRNA. Larger sample sizes of injected eggs are required to overcome the high mortality rates in the experiments. Additionally, female-only offspring from the Ag strain (reported in chapter 1) can be used for microinjection. Microinjection of functional Mdmd mRNA in female-only embryos will increase the efficiency to generate sex-reversed males. A second approach would be to repeatedly express MdmdV throughout the developmental period of M. domestica, by generating MdmdV transgenic flies via piggyBac transformation. If MdmdV is the only male-determining gene performing the male-determining function, repeated expression of MdmdV should transform genotypic females into males. The cloning of a pBac[3×P3-EGFP, hsp70-MdmdV] transgene is described in Box 4.1.

There are several reasons for determining whether Mdmd performs the male-determining function and is solely sufficient for male development. First, it would show that a single gene that is expressed during early embryogenesis can be responsible for male development. Second, as it is quite common in insects that male sex is determined by a dominant male-determining factor (Marín and Baker, 1998; Beukeboom and Perrin, 2014), male-determining factors in other insect species may act the same as Mdmd. Third, knowledge of the male-determining mechanism may be useful in pest control. It may help to generate transgenic insects that only produce male progeny to, for example, improve the sterile insect technique (SIT) that relies on mass release of males only. Thus, clarifying the role of sex-determining genes in the M. domestica sex determination pathway will provide fundamental insights into insect sex determination systems, but may also have applied relevance.

4.6 Acknowledgements

I would like to thank Dr. Daniel Bopp for providing the opportunity to perform microinjections in his lab. I thank Tea Kohlbrenner and Svenia Heinze for help with practical work. I am very grateful to Martijn A. Schenkel, Peter K. Hoitinga, Marcela Buricova and Aurore Panel for their valuable suggestions on this manuscript and to Martijn A. Schenkel for performing the χ² test for table 1. I also want to thank Akash Sharma for providing the four orphan reads of the male-biased sequences, and Natalia Siomava for providing of anti-sense probe for the apterous gene and Marita Büscher for providing the DAPI.
4.7 Appendix

4.7.1 Primer sequences

MdmdF:
5’-ATTTAGGTGACACTATAGCAACAAAAATATGAATGCCACCCGAC-3’
MdmdR1:
5’-GAAATTAATACGACTCACTATAGGCTTAGTCATTAGTGCTCCCTGGG-3’
F1: 5’-CACTCGTTTCAGAACTTTGGAAGAATGGACGCCAGAAAAAGG-3’
R4: 5’-GTGTTTGATAGCAAGAATTAGGAGT-3’
MdncmF1:
5’-AAGCTATTTAGGTGACACTATAGGAGAAGAATGGACGCCAGAAAAAGG-3’
MdncmR1:
5’-GAAATTAATACGACTCACTATAGGCTTAGTCATTAGTGCTCCCTGGG-3’

4.7.2 Buffers

1L 10×PBS: add 0.07 M Na₂HPO₄, 1.4 M NaCl, 0.03 M KH₂PO₄, dissolve the reagents in 800 mL H₂O and adjust the pH to 7.4 then add H₂O to 1 L, followed by autoclaving.
PBT: 1×PBS and 0.1% Triton.
4% PFA: add 4 g of EM grade paraformaldehyde to 50 mL of H₂O. Add 1 mL of 1 M NaOH and stir gently on a heating block at ~60°C until the paraformaldehyde is dissolved. Add 10 mL of 10×PBS and allow the mixture to cool to room temperature. Adjust the pH to 7.4 with 1 M HCl (~1 mL), then adjust the final volume to 100 mL with H₂O. Filter the solution through a 0.45 μm membrane filter to remove any particulate matter. Make the paraformaldehyde solution fresh prior to use, or store in aliquots at -20°C for several months.
20×SSC: add 175.3 g NaCl, 88.2 g Trisodium citrate.2H₂O, dissolve the chemicals in 800 mL dd H₂O and adjust pH to 5.5 with HCl, add dd H₂O to 1 L.
Hybe-A solution: add 50 mL Formamid, 25 mL 20×SSC, 25 mL deionized water, then add 500 μL 20 mg/mL Yeast RNA, 100 μL 50 mg/mL Heparin, 2 mL sonicated salmon sperm DNA (10 mg/mL) that should be boiled for 10 minutes and 3 minutes on ice before adding.
NBT buffer: 1 mL 1 M Tris pH 9.5, 500 μL 1 M MgCl₂, 200 μL 5 M NaCl, 50 μL 20% Triton, add ddH₂O to 10 mL.
Resuspension buffer: add 500 μL Formamid, 5 μL 20% Tween 20, 250 μL 20×SSC, 0.4 μL 50 mg/mL Heparin and 245 μL DEPC H₂O.
Squishing buffer: add 10 mM Tris (pH 8.2), 1 mM EDTA; 25 mM NaCl and 0.02 mg/mL proteinase K.
10× MOPS (1 L): 83.7 g of MOPS (3-Morpholinopropane-1-sulfonic acid), 33.3 µl of NaAc and 20 µl of 0.5 M EDTA (pH 7.0).

Sodium hypochlorite solution: 1:10 dilution of 14% NaOCl with tap water.

Ringer’s solution (pH 7.3-7.4) (1 L): dissolve 7.2 g NaCl, 0.17 g CaCl₂ and 0.37 g KCl in Milli-Q water and bring the final volume to 1 L. pH was adjusted to 7.3-7.4. Once thoroughly dissolved, the solution was filter through a 0.22 µm filter and aliquoted into single-use volumes (25-50 mL), followed by autoclaving.
Box 4.1
Transgene construction for repeated expression of *Mdmd* in *Musca domestica*

Microinjection of *Mdmd* with capped, polyadenylated RNA did not yield any masculinised flies, which may be due to various technical reasons, such as transient decay of the mRNA. An alternative approach towards answering the question whether expression of *Mdmd* is sufficient to turn genotypic females into males, would be to use *piggyBac* germline transformation to repeatedly express *Mdmd* during the whole life-cycle of the housefly. The *piggyBac*-EGFP vector-marker system was effectively used in *M. domestica* before (Hediger et al., 2001, 2004, 2010). Here, I describe the cloning of a pBac[3×P3-EGFP, hsp70-*Mdmd*] transgene. This transgene will be used in germline transformation to generate transgenic flies, in which *Mdmd* can be repeatedly expressed under the control of the hsp70 promoter. Such experiments will help to assess the masculinising activity of *Mdmd*.

1. Molecular cloning of the *Mdmd* cDNA to pSLfaHSfa

To achieve repeated expression of *Mdmd*, a pBac[3×P3-EGFP, hsp70-*Mdmd*] transgene was constructed for germline transformation. The *Mdmd* cDNA was first cloned to the versatile cloning shuttle vector pSLfaHSfa as follows (Box 4.1 fig. 1; Ramos et al., 2006). A restriction site for NotI (GC|GGCCGC) was supposed to be introduced upstream of the *Mdmd* coding region by PCR with the primers 31_GSP2b-Dra52_GSP3_consensus_F1 and M13R, yielding a fragment of 3.7kb (Box 4.1 fig. 2), which was supposed to contain the SpeI (A|CTAGT) and NotI restriction sites at opposite ends. Next, the enzymes SpeI and NotI were chosen to digest the purified PCR product and ligated into the pSLfaHSfa vector, which was digested by the enzymes XbaI (T|CTAGA) and NotI. As SpeI and XbaI have compatible cohesive ends, the pCR®II-*Mdmd* construct that was digested by SpeI and NotI and the pSLfaHSfa vector that was digested by XbaI and NotI can be ligated together.
Box 4.1 figure 1: Cloning strategy to obtain pSLfaHSfa+MdmdV. A restriction site for NotI was supposed to be introduced upstream of the MdmdV coding region by PCR with primers 31_GSP2b-Dra52_GSP3_consensus_F1 and M13R. The enzymes SpeI and NotI were chosen to digest the PCR product and ligate into the pSLfaHSfa vector, which was digested by the enzymes XbaI and NotI. SpeI and XbaI have compatible cohesive ends after digestion. Primer combination GSP1 and M13R was chosen to perform colony PCR for selecting positive colonies.

Box 4.1 figure 2: PCR of pCR®II+MdmdV construct with the primers M13R and 31_GSP2b-Dra52_GSP3_consensus_F1 yielded a DNA fragment of 3.7kb.
The pSLfaHSfa vector was digested with XbaI and NotI, yielding a 4.1kb target fragment (Box 4.1 fig. 3, lane 1). Next, the 3.7kb fragment from the previous PCR amplification was digested by SpeI and NotI, yielding a 3.6kb target fragment (Box 4.1 fig. 3, lane 2). The 4.1kb and 3.6kb fragments were purified for ligation.

The Mdmv cDNA was successfully ligated into the pSLfaHSfa shuttle vector. Successful ligation was checked with colony PCR, in which a fragment of 831bp was successfully amplified with primers GSP1 in the Mdmv cDNA and M13R in the pSLfaHSfa vector (Box 4.1 fig. 1). Colonies from #7 and #8 showed the target fragment in the colony PCR (Box 4.1 fig. 4). Sequencing of #8 confirmed successful cloning and revealed that there was no nucleotide mutation generated throughout the cloning process, but a wrong NotI recognition sequence (CGCCGGCG instead of GCGGCCGC) had accidentally been introduced upstream of the Mdmv coding region. Theoretically, the 3.7kb fragment digested by SpeI and NotI should not have been ligated into the pSLfaHSfa vector, which was digested by XbaI and NotI. Colony #8 shows, however, that the insert was inserted into the vector uncut at its 5' end including all the sequence of primer 31_GSP2b-Dra52_GSP3_consensus_F1. As the insertion of the incorrect NotI recognition sequence including the rest of the primer did not alter the expected Mdmv coding sequence, and did not introduce any extra ATG upstream of the Mdmv coding sequence, the Mdmv cDNA was still successfully cloned into the expression shuttle vector.
Box 4.1 figure 4: Construction of pSLfaHSfa+MdmdV. Successful ligation yielded a 831bp fragment in the colony PCR and colonies from #7 and #8 showed the target fragment.

2. Construction of the pBac[3×P3-EGFP; hsp70-MdmdV] transgene

The pBac[3×P3-EGFP; hsp70-MdmdV] transgene was successfully constructed as follows (Box 4.1 fig. 5). The pSLfaHSfa+MdmdV construct was digested by FseI (GGCCGG|CC) and Ascl (GG|CGCGCC), yielding two fragments of 3.1kb and 4.6kb (Box 4.1 fig. 6). The pBac[3×P3-EGFPafm] vector was digested with the same enzymes, yielding a 6.5 kb target fragment. The 4.6kb and 6.5kb fragments were purified for ligation.

Box 4.1 figure 5: Cloning strategy to obtain pBac[3×P3-EGFP; hsp70-MdmdV]. The enzymes FseI and Ascl cut the hsp70-MdmdV and after that it was ligated into pBac[3×P3-EGFPafm], which was digested with the same enzymes.
Restriction digestion of pSLfaHSfa+MdmdV and pBac[3×P3-EGFPafm] vector. Lane 1 contains FseI and Ascl digestion of pSLfaHSfa+MdmdV that yielded fragments of 3.1kb and 4.6kb. Lane 2 contains FseI and Ascl digestion of the pBac[3×P3-EGFPafm] vector that yielded a fragment of 6.5kb. The 4.6kb and 6.5kb fragments with arrows were purified for ligation.

The transgene pBac[3×P3-EGFP; hsp70-MdmdV] was successfully constructed (Box 4.1 fig. 7). Successful ligation was checked with colony PCR, in which fragments of 914bp were successfully amplified with primers pBacF2 and GSP1 (Box 4.1 fig. 5). The plasmids from two of these positive colonies were extracted and the size of inserted DNA fragments was checked by EcoRI digestion. There are three EcoRI cutting sites in the hsp70-MdmdV and two EcoRI cutting sites in the pBac[3×P3-EGFPafm] vector (Box 4.1 fig. 5). I therefore obtained six differently sized fragments by EcoRI digestion: 6.2kb, 2.7kb, 930bp, 745bp, 426bp and 107bp, which confirmed candidates #5 and #6 (Box 4.1 fig. 7). Sequencing of the insert in #5 revealed that there was no nucleotide mutation generated throughout the cloning process. Hence, the transgene pBac[3×P3-EGFP; hsp70-MdmdV] was successfully cloned.
Box 4.1 figure 7: Construction of pBac[3×P3-EGFP; hsp70-MdmdV] transgene. A: Colony PCR: successful ligation yielded fragments of 914bp in the colony PCR and some colonies among #1, #2, #5, #6, #9, #10, #11, #12, #13, #14, #15, #16, #17, #18, #19, #20, #21 and #22 showed target fragments. B: EcoRI test digestion yielded six differently sized fragments: 6.2kb, 2.7kb, 930bp, 745bp, 426bp and 107bp and candidates #5 and #6 were confirmed (the 107bp fragment is not visible in the gel picture because it ran out of the gel).

I have reported how I successfully constructed a pBac[3×P3-EGFP, hsp70-MdmdV] transgene for repeated expression of MdmdV in germline transformation. I used a versatile two-step cloning procedure to get the transgenic construct pBac[3×P3-EGFP; hsp70-MdmdV]. The piggyBac element is an efficient transposable vector in insects and the piggyBac-EGFP vector-marker system has already been used in M. domestica (Hediger et al., 2001, 2004, 2010) (Box 4.1 fig. 8A). The green fluorescent protein (GFP) derived from the jellyfish Aequorea victoria is a universal gene expression marker that is active in various organisms (Tsien, 1998). The expression of EGFP (an enhanced GFP variant) is driven by an artificial promoter 3×P3 (Sheng et al., 1997; Heim et al., 1995; Berghammer et al., 1999). Transformed individuals can be identified by GFP expression in their pigmentless eyes. The pSLfaHSfa vector is a versatile cloning
shuttle vector with an hsp70 promoter upstream of its polylinker site and an hsp70 3’UTR downstream of it (Box 4.1 fig. 8B). Importantly, the pSLfaHSfa vector contains several hexa-cutters and NotI, which enables the assembly of complicated constructs (Ramos et al., 2006). Additionally, it also contains rare octa-cutter restriction sites Ascl and FseI located at either end of hsp70 site (Ramos et al., 2006), which allows for target genes to be inserted into the pSLfaHSfa vector to be cloned into different transformation vectors with different markers by double digestion with FseI and Ascl, followed by ligation.

Finding unique digestion sites in the *Mdmd* cDNA and screening the possible constructs is very complicated and laborious due to the 3.5kb length of the *Mdmd* ORF. The two-step cloning procedure as used here avoids re-cloning of such complicated constructs. By generating the pSLfaHSfa+*Mdmd* construct, *Mdmd* can be easily cloned into different transformation vectors with different markers by double digestion with FseI and Ascl, followed by ligation.

I now have a transformation vector that can be used to express *Mdmd* during the whole life cycle of the housefly. With this I can test whether *Mdmd* performs the male-determining function and whether it is sufficient for male development. If repeated expression of *Mdmd* under the control of hsp70 promotion would
turn genotypic females to males, it will prove that \textit{Mdmd} \textsuperscript{V} is sufficient for male development. If repeated expression of \textit{Mdmd} \textsuperscript{V} would have a partial masculinisation effect, it may indicate that \textit{Mdmd} \textsuperscript{V} is not sufficient for male development. If repeated expression of \textit{Mdmd} \textsuperscript{V} in female germline would fail to turn genotypic females to males, it would demonstrate that even though \textit{Mdmd} \textsuperscript{V} has an open reading frame, it does not perform the male-determining function, or at least not by itself. These transformation studies will help to clarify the role of sex-determining genes in the \textit{M. domestica} sex determination pathway, as well as provide insights into insect sex determination systems.

3. Further details on methodology

A transgene pBac[3×P3-EGFP, hsp70-\textit{Mdmd} \textsuperscript{V}] was first constructed for generating transgenic flies. I cloned \textit{Mdmd} \textsuperscript{V} to the pSLfaHsfa vector from a pCR\textsuperscript{®}II+\textit{Mdmd} \textsuperscript{V} construct. Two unique enzymes NotI and XbaI were chosen to digest the pSLfaHsfa vector and unique enzymes NotI and SpeI to digest the pCR\textsuperscript{®}II+\textit{Mdmd} \textsuperscript{V} construct. Because there is no unique NotI recognition site in the pCR\textsuperscript{®}II+\textit{Mdmd} \textsuperscript{V} construct, a restriction site for NotI (GC|GGCCGC) was supposed to be introduced upstream of the \textit{Mdmd} \textsuperscript{V} coding reagon by PCR with the following concentrations and conditions: 100 ng pCR\textsuperscript{®}II+\textit{Mdmd} \textsuperscript{V} template DNA, 0.5 µL 10 µM 31\textsuperscript{GSP2b-Dra52}\textsuperscript{GSP3_consensus_F1}, 0.5 µL 10 µM M13R, 2 µL 2.5 mM dNTP, 5 µL 5×Phusion Buffer and 0.5 µL Phusion enzyme in a total volume of 25 µL. The NotI restriction site is supposed to be included in the sequence of 31\textsuperscript{GSP2b-Dra52}\textsuperscript{GSP3_consensus_F1}. PCR was performed by denaturation at 94°C for 2 min, then 25 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 4:30 min, and lastly extension at 72°C for 10 min. PCR products were analysed on a 1% agarose/EtBr gel. The target fragment was purified with the NucleoSpin\textsuperscript{®} Gel and PCR clean-up kit from Macherey-Nagel. SeqLab (Göttingen, Germany) carried out sequencing of the candidate fragments with the vector primers M13F and M13R combined with 46\textsuperscript{GSP2b-Dra52-F-GSP4b-R-F}, 46\textsuperscript{GSP2b-Dra52-F-GSP4b-R-R} and R4.

After checking the target fragment by sequencing and verifying that no mutation occurs throughout the sequence, the target fragment was double digested with NotI and SpeI from NEB (Massachusetts, United States) with the following concentrations and conditions: 1 µg DNA from target fragment, 1 µL SpeI-HF\textsuperscript{®} (20 u/µL), 1 µL NotI-HF (20 u/µL), 2 µL 10×CutSmart\textsuperscript{®} Buffer in a total volume of 20 µL; followed by incubating at 37°C for 3 hrs. The enzymes NotI-HF (20 u/µL) and XbaI (20 u/µL) from NEB (Massachusetts, United States) were used to digest the vector pSLfaHsfa with the same concentrations and conditions shown above. The digestions were checked on a 1% agarose/EtBr gel and the target
fragments isolated by the NucleoSpin® gel and PCR clean-up kit from Macherey-Nagel (Düren, Germany). Ligation was performed with the following concentration and conditions: 300 ng insert DNA, 100 ng vector DNA, 1 µL T4 ligation buffer in a total volume of 9 µL, 1 µL T4 ligase from NEB (Massachusetts, United States) was added to the reaction to reach the final volume of 10 µL. The ligation was performed at 16°C overnight. The construct was used to transform competent *E. coli* DH5α.

After transformation, colony PCR was performed under the following conditions and concentrations: 0.2 µL 10 µM GSP1, 0.2 µL 10 µM M13R, 0.8 µL 2.5 mM dNTP, 1 µL 10×Advantage 2 PCR Buffer and 0.1 µL Advantage 2 Polymerase Mix (50×) in a total volume of 10 µL. A single colony was added in each reaction separately. Colony PCR was performed by denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 1 min, and lastly extension at 72°C for 10 min. PCR products were analysed on a 1% agarose/EtBr gel. Positive colonies were cultured in Luria-Bertani (LB) medium that contain 100 µg/mL ampicillin at 37°C overnight. Plasmids from positive colonies were extracted the following day and the size of inserted DNA fragments was checked by NotI-HF® (NEB, Massachusetts, United States) digestion. LGC Genomics (Berlin, Germany) carried out sequencing of the candidate fragments with the vector primers M13F and M13R combined with 46-GSP2b-Dra52-F-GSP4b-R-F, 46-GSP2b-Dra52-F-GSP4b-R-R, R4.

After *Mdmvl* was cloned to the pSLfaHsfa polylinker site, hsp70-*Mdmvl* was cloned to the pBac[3×P3-EGFPafm] vector by double digestion with Ascl (10 u/µL) and FseI (2 u/µL) from NEB (Massachusetts, United States) with the same concentrations and conditions as above. The digestions were incubated at 37°C for 1 hr, followed by checking on a 1% agarose/EtBr gel. The target fragments were isolated by NucleoSpin® gel and PCR clean-up kit from Macherey-Nagel (Düren, Germany). Ligation was performed as above. Colony PCR was performed with primer combination GSP1 and pBacF2. The size of inserted DNA fragments in plasmids from positive colonies was checked by EcoRI-HF® (NEB, Massachusetts, United States) digestion. LGC Seqlab (Göttingen, Germany) carried out sequencing of the candidate fragments from positive plasmids with the vector primer pBacF2 combined with 31_GSP2b-Dra52_GSP3_consensus_F1, 46-GSP2b-Dra52-F-GSP4b-R-F, cDNA_F1_MII_MV, 12-GSP1-9-F-GSP4b-R-R, R4 and GSP4b.

Sequencing data from cloning were analysed with “Geneious” (Kearse et al., 2012). Nucleotide multiple alignment was used in the data processing.
4. Acknowledgements

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5. Appendix

5.1 Primer sequences

31_GSP2b-Dra52_GSP3_consensus_F1:
5’-TACGCCGGCCCTGACAACAAAAATATGAATGC-3’
M13F: 5’-GTAAAAACGACGCGCCATG-3’
M13R: 5’-CAGGAACAGCTATGAC-3’
46-GSP2b-Dra52-F-GSP4b-R-F: 5’-CCAGGACAAGGACAATCGACTAAGACG-3’
46-GSP2b-Dra52-F-GSP4b-R-R: 5’-AAGAACTTGATGAGGACGACGAGGTGC-3’
GSP1: 5’-TCTACTGGGTGTTTATTTGATCCTGGCTG-3’
R4: 5’-GTGTGATGATAAGAATTAGGAGT-3’
pBacF2: 5’TAGGCGAGTCTCTGACTGAACATTGTC-3’
cDNA_F1_MIII_Mv: 5’TCTTAACAGTCTTAAATTCAATCGCCGACTG-3’
12-GSP1-9-F-GSP4b-R-R: 5’TCTATCGTTGCTGACTTCTGCTTC-3’
GSP4b: 5’-AGTGAAATTAAAGACGCGAGACGGACG-3’