Identification of Dmrt2a downstream genes during zebrafish early development using a timely controlled approach

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Abstract

Background: Dmrt2a is a zinc finger like transcription factor with several roles during zebrafish early development: left-right asymmetry, synchronisation of the somite clock genes and fast muscle differentiation. Despite the described functions, Dmrt2a mechanism of action is unknown. Therefore, with this work, we propose to identify Dmrt2a downstream genes during zebrafish early development.

Results: We generated and validated a heat-shock inducible transgenic line, to timely control dmrt2a overexpression, and dmrt2a mutant lines. We characterised dmrt2a overexpression phenotype and verified that it was very similar to the one described after knockdown of this gene, with left-right asymmetry defects and desynchronisation of somite clock genes. Additionally, we identified a new phenotype of somite border malformation.

We generated several dmrt2a mutant lines, but we only detected a weak to negligible phenotype. As dmrt2a has a paralog gene, dmrt2b, with similar functions and expression pattern, we evaluated the possibility of redundancy. We found that dmrt2b does not seem to compensate the lack of dmrt2a. Furthermore, we took advantage of one of our mutant lines to confirm dmrt2a morpholino specificity, which was previously shown to be a robust knockdown tool in two independent studies.

Using the described genetic tools to perform and validate a microarray, we were able to identify six genes downstream of Dmrt2a: foxj1b, pxdcb, cxcl12b, etv2, foxc1b and cyp1a.

Conclusions: In this work, we generated and validated several genetic tools for dmrt2a and identified six genes downstream of this transcription factor. The identified genes will be crucial to the future understanding of Dmrt2a mechanism of action in zebrafish.

Keywords: Dmrt2a, dmrt2a transgenic, Dmrt2b, TALEN mutants, Microarrays, Zebrafish

Background

The DMRT (Doublesex and Mab3 Related Transcription factors) family of zinc finger like transcription factors has been classically associated with sexual determination and differentiation. Although these functions remain fairly conserved in the different animals studied so far, during evolution different members of this family acquired different functions, such as nervous system development, somitogenesis and left-right asymmetry establishment [1].

When considering the DMRT family, Dmrt2a is particularly notorious because it was the first DMRT protein to be identified with a role apart from sexual determination and differentiation. Meng et al. were the first to describe dmrt2a expression pattern in the somites, in zebrafish and mouse, and to propose its role in somitogenesis [2]. Later, dmrt2a was found in the Left-Right Organiser (LRO) of zebrafish and chicken, and was found to be necessary not only for somitogenesis but also to the synchronisation of the somite clock genes, left-right asymmetry and more recently, to fast muscle differentiation [3–6]. Moreover, a fish-specific paralog gene – dmrt2b – was also identified with similar...
roles regarding somitogenesis and left-right asymmetry establishment [7, 8].
In mouse, DMRT2 was only associated with somitogenesis-related processes. It was proposed that this transcription factor could affect extracellular matrix components such as LAMININ-1 and, it was further demonstrated that DMRT2 could act in a PAX3/DMRT2/MYF5 regulatory cascade at the onset of myogenesis [9, 10].
On the other hand in zebrafish, several roles were described for Dmrt2a, but little is known about its targets and signalling pathway. Until now, only two players are known to interact with \textit{dmrt2a}, at the level of its 3'UTR: Celf1 and \textit{miR-203a} [5, 6]. Celf1 is an RNA-binding protein that promotes \textit{dmrt2a} mRNA decay. Upon overexpression of \textit{celf1}, \textit{dmrt2a} mRNA levels are decreased, and problems in the synchronisation of the somite clock genes and in left-right asymmetry establishment arise [5]. miR-203a is a microRNA, which also regulates \textit{dmrt2a} mRNA levels, being needed to ensure correct fast muscle development [6].
In this work, our main goal was to identify Dmrt2a downstream genes in order to clarify how this transcription factor controls so many different developmental processes in zebrafish. In order to accomplish this goal, we generated a heat-shock inducible transgenic line that allowed us to induce a timely controlled \textit{dmrt2a} overexpression, and using the TALEN technology, we generated \textit{dmrt2a} stable mutant lines. Taking advantage of one of the \textit{dmrt2a} mutant lines, we were able to validate the specificity of the previously used \textit{dmrt2a} morpholino (MO) [3, 5]. Using our tools, we performed and validated a microarray experiment. We identified \textit{foxj1b}, \textit{pxdc1b}, \textit{cxl12b}, \textit{etv2}, \textit{foxc1b} and \textit{cyp1a} as genes downstream of Dmrta2. Our findings give the first steps in order to understand Dmrta2 mechanism of action during zebrafish early development.

**Results**

**Generation of a heat-shock inducible transgenic line to timely overexpress \textit{dmrt2a}**
We generated a heat-shock inducible transgenic line Tg(hsp70:HA-\textit{dmrt2a}) (Fig. 1a) to overexpress \textit{dmrt2a} in a timely controlled manner. We induced a heat-shock (Fig. 1b) at bud-stage given that this time-point coincides with \textit{dmrt2a} early expression (Fig. 1c), marks the beginning of somitogenesis [11] and precedes the formation of zebrafish LRO [12].
To ensure we had a homogenous transgenic population, we selected the fish with only one copy of the transgene (Fig. 1d). We confirmed that after a heat-shock, the HA-Dmrt2a fusion protein was produced with the correct molecular weight (Fig. 1e) and translocated into the nucleus (Fig. 1f). This translocation was controlled by the endogenous nuclear localisation signal contained within Dmrt2a DM domain [13].

**Transient increase of \textit{dmrt2a} expression produces a similar phenotype to \textit{dmrt2a} loss-of-function**
We used the Tg(hsp70:HA-\textit{dmrt2a}) to investigate the impact of transiently overexpressing \textit{dmrt2a} during the

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**Fig. 1** Generation and characterisation of the Tg(hsp70:HA-\textit{dmrt2a}) line. **a** Transgenic construct design. **b** Heat-shock protocol: embryos were raised at 28 °C and the heat-shock performed for 30 min at 39 °C. **c** qPCR analysis showing that \textit{dmrt2a} transcripts are present from bud-stage until 20-somite stage. **d** Southern blot hybridisation using a 32P-labelled γ-crystallin probe showing F0 fish with multiple bands (C+, positive control), wildtype fish (C−, negative control), and F1 generation fish with only one copy of the transgene or multiple copies. **e** The HA-Dmrt2a protein is produced with the correct molecular weight (57 kDa). **f** The fusion protein HA-Dmrt2a (red) co-localises with the nucleus (blue). h: hours, min: minutes, ss: somite stage. Scale bar: 30 μm
developmental processes known to be regulated by this gene: left-right asymmetry and somite formation [3, 5].

In the context of left-right asymmetry establishment, dmrt2a overexpression led to an increased bilateral expression of the laterality markers, spaw and pitx2, when compared to controls (Fig. 2a, b). As Nodal signalling promotes the left-jog of the heart tube [14] and given that spaw and pitx2 became bilateral upon dmrt2a overexpression, we expected an equivalent percentage of no-jog heart phenotype. However, we obtained an increased percentage of right-jog phenotype, when compared to controls (Fig. 2c). Although bilaterally expressed, small differences in spaw levels, between the left and right sides of the lateral plate mesoderm, could be sufficient to drive an asymmetric heart jogging. During heart tube morphogenesis, jogging to the left side is followed by looping to the right side of the embryo (i.e., D-loop). When compared to controls, we obtained a higher percentage of no-loop and S-loop (i.e., left-sided loop) heart phenotypes after dmrt2a overexpression, showing a lack of agreement between jogging and looping (Fig. 2d). These results further support the idea that heart looping can be a Nodal independent mechanism [15].

In the context of bilateral somite formation, we obtained more embryos with a desynchronised expression of the somite clock genes, deltaC, her1 and her7 after dmrt2a overexpression, when compared to controls (Fig. 3a-c). To evaluate the impact of this desynchronisation in somitogenesis, we evaluated pcdh8, mespb, myod1 and cb1045 expression patterns. All these markers revealed defects in somite morphogenesis (Fig. 4a-d) that can be due to poorly defined or absent intersomitic borders, as we observed (Fig. 4e).

Therefore, dmrt2a overexpression produces a similar phenotype to the one previously described when this gene is knockdown [3, 5], suggesting a need to fine tune dmrt2a levels during early development. Additionally, we observed a robust new phenotype in the formation of somite boundaries.

A mild to negligible phenotype is observed in dmrt2a mutants

In order to produce stable dmrt2a mutant lines, we used the TALEN technology (Fig. 5a and Additional file 1: Figure S1a, b). We obtained two different mutants: dmrt2aΔ100, which lacks the 3’end of the 5’UTR, the start codon and part of the first exon spanning a 100 bp
Fig. 3 Characterisation of dmrt2a overexpression phenotype: somite clock genes. **a** deltaC (8-somite stage). **b** her1 (8-somite stage). **c** her7 (8-somite stage). The embryos shown illustrate the different phenotypes obtained. Sym: symmetric, S-Asym: slightly asymmetric, Asym: asymmetric.

Fig. 4 Characterisation of dmrt2a overexpression phenotype: somite differentiation and formation markers. **a** Somite differentiation gene pcdh8 (8-somite stage). **b** Somite boundary gene mespb (8-somite stage). **c** Muscle differentiation marker myod1 (14-somite stage). **d** Somite differentiation gene cb1045 (24 hpf). The embryos shown illustrate the different phenotypes obtained. **e** F-actin staining in control (n = 40/42) and in Tg(hsp70:HA-dmrt2a) after dmrt2a overexpression (n = 22/51) where lack of intersomitic borders is observed (i, ii). Embryos between 8 and 10-somite stage were used. Sym: symmetric, Asym: asymmetric, BD: border defect, hpf: hours post-fertilisation. Scale bar: 45 μm (e) and 20 μm (zoomed panels i and ii).
region; and dmrt2aΔ14, which also lacks the start codon and has a 12 bp duplication in the first exon (Fig. 5b). dmrt2a mutants in homozygosity were viable and were used to characterise the loss-of-function phenotype. We observed that dmrt2aΔ14−/− had a mild phenotype regarding spaw expression pattern and dmrt2aΔ100−/− only had a very mild phenotype regarding heart jogging (Fig. 6a, b). In both cases, her7 expression pattern was unaffected (Fig. 6c).

As dmrt2a has a paralog gene – dmrt2b – with a similar expression pattern in somites and with described roles in somitogenesis and left-right asymmetry establishment [7, 8], we wanted to verify if, in the absence of dmrt2a, dmrt2b could compensate its lack. Therefore, using the TALEN technology, we also produced a dmrt2b mutant, which has an early stop codon (p.E22_W23insX, Fig. 5a, b and Additional file 1: Figure S1c). Also in homozygosity, dmrt2b mutants were viable and presented an even milder phenotype than dmrt2a mutants in the different evaluated markers (Fig. 6a-c). We proceeded with the generation of double mutants: dmrt2aΔ100;dmrt2b and dmrt2aΔ14;dmrt2b. When analysing the double mutants, we observed that: (1) the spaw phenotype in dmrt2aΔ14−/−;dmrt2b−/− was milder than in the single dmrt2aΔ14−/− mutant, and (2) the heart jogging phenotype present in dmrt2aΔ100−/− single mutant, disappeared in the double mutant context (Fig. 6d, e). In both cases, myod1 expression pattern was unaffected (Fig. 6f). As the left-right asymmetry phenotype observed was not consistent between single and double mutants and, as previously reported, zebrafish wildtype embryos can have up to 10% left-right asymmetry defects [16], we considered our phenotypes negligible.

The weak or negligible phenotype observed in our mutant lines can have several explanations, such as the presence of alternative ATGs, the production of Dmrt2 proteins with residual function or the activation of compensatory genes. Regarding the first hypothesis, we evaluated all the possible alternative ATGs present from the 5′ end of the 5′ UTR of dmrt2a until the 3′ end of the DM domain, which contains the two DNA-binding domains and the crucial amino acid residues for nuclear translocation (Fig. 5c) [13]. We were able to identify only one ATG in dmrt2a mutants, located near the 3′ end of the DM domain that maintained dmrt2a reading frame (Fig. 5c). If this ATG was used, the DM domain would be truncated and consequently, would lack the residues shown to be crucial for nuclear translocation. As Dmrt2a is a transcription factor, we would expect that the inability to enter the nucleus would render this protein dysfunctional. Regarding dmrt2b mutants, we were able to find an alternative
ATG, after the premature STOP codon, which enabled the production of a functional DM domain (Fig. 5c). However, there is no guarantee that this ATG is in an appropriate genomic environment to allow its use.

In the absence of specific antibodies against Dmrt2a and Dmrt2b to evaluate the production of these proteins, we cannot be sure that we have true null mutants. Therefore, we used an indirect approach: we evaluated the levels of both transcripts based on the assumption that, if dmrt2a and dmrt2b transcripts are dysfunctional they should be degraded.

The results we obtained from this evaluation were the following: (1) in single dmrt2a mutants and in double mutants, dmrt2a transcript was increased; (2) in single dmrt2b mutants, its transcript was increased and dmrt2a transcript was unaffected; (3) in single dmrt2a mutants and in double mutants, dmrt2b transcript was not affected (dmrt2aΔ100−/− and dmrt2aΔ14−/−;dmrt2b−/−) or was only slightly affected (dmrt2aΔ14−/− and dmrt2aΔ14−/−;dmrt2b−/−) (Fig. 7). These results show that dmrt2a and dmrt2b transcripts are under a negative feedback loop regulating their own expression, which suggests a deficiency in the production of Dmrt2a and Dmrt2b proteins. However, we still cannot assume that the mutations obtained produce null alleles. Our data also corroborates the lack of redundancy between dmrt2a and dmrt2b, further supporting previously published results [8].

dmrt2a-MO proves to be specific and non-toxic

At this moment, we lack specific antibodies to confirm if the different mutants obtained are true nulls and, assuming that a compensatory mechanism is taking place, we have yet to identify possible candidate gene(s) for this role. Nevertheless, we took advantage of one of our mutant lines to re-validate dmrt2a-MO [3, 5].

**Fig. 6** Characterisation of the single and double dmrt2a and dmrt2b mutants phenotype. a, b, c Characterisation of dmrt2aΔ100−/−, dmrt2aΔ14−/− and dmrt2b−/− mutant phenotype using: (a) left-sided gene spaw (20-somite stage), (b) myl7 for heart jogging at 30 hpf and (c) her7, a cycling gene (8-somite stage). d, e, f Characterisation of dmrt2aΔ100−/−;dmrt2b−/− and dmrt2aΔ14−/−;dmrt2b−/− double mutants phenotype using: (d) left-sided gene spaw (20-somite stage), (e) myl7 for heart jogging at 30 hpf and (f) myod1, a muscle differentiation marker (14-somite stage). L: left, R: right, B: bilateral, NAE: no anterior expansion, Sym: symmetric, S-Asym: slightly asymmetric, Asym: asymmetric, WT: wildtype, hpf: hours post-fertilisation.
To accomplish this, we used the dmrt2aΔ100−/− background, which lacks 13 nucleotides of the dmrt2a-MO binding site (Fig. 8a). As the morpholino lacks it preferred binding site, it is the ideal situation to evaluate if it can bind non-specifically to other regions in the genome. To perform this evaluation, we injected dmrt2a-MO in the mutant background and compared it with wildtype embryos injected in the same conditions. After injecting dmrt2a-MO in a wildtype background, we observed the expected phenotype in myod1 and spaw expression (Fig. 8b, c). In contrast, this phenotype was absent or extremely reduced when injecting dmrt2a-MO in the mutant background (Fig. 8b, c). Our results suggest that, in the evaluated conditions, dmrt2a-MO does not have detectable off-target effects. Additionally, we observed that the toxicity marker – p53 – was not significantly affected after dmrt2a-MO injection (Figs. 8d, d’ and 9b, c for procedure details). As dmrt2a-MO seems to be specific, without off-targets and non-toxic, we decided to use it as a preferential loss-of-function tool.

**Genome wide approach to identify Dmrt2a downstream genes**

In the context of the Tg(hsp70:HA-dmrt2a), the HA-Dmrt2a fusion protein peaks between 1 h 30 min and 2 h post-heat-shock (Additional file 2: Figure S2). Given that our goal was to identify Dmrt2a most immediate downstream genes we decided to collect embryo samples between 2 h (~3-somite stage) and 2 h 30 min (~4-somite stage) after heat-shock (Fig. 9a). Embryo samples were collected using a microdissection protocol. We microdissected Region 1 (containing somites) and Region 2 (containing tailbud/LRO) which are dmrt2a expressing territories (Fig. 9c). These samples were used in a microarray analysis.

In the microarray data (Fig. 9d, e and Additional figure 3: Figure S3a, b), dmrt2a levels are increased as expected. However, dmrt2b was significantly increased in Region 2 (Fold change (FC) = 2.63, P = 0.000008, False Discovery Rate (FDR) = 0.0212) but not in Region 1 (FC = 1.3, P = 0.000416, FDR = 0.0677). This could be due to the higher physiological expression levels of dmrt2a in somites (Region 1) when compared to tailbud/LRO (Region 2).

DMRT proteins were described to act as activators, repressors or both [17, 18]. Our microarray analysis revealed that Dmrt2a seems to act mainly as a repressor during early development.

The analysis of the microarray showed some genes that could be expected to be downstream of Dmrt2a, such as myf5 (myogenesis related [10]) and foxj1b (left-right asymmetry related [19]). Interestingly, a category of genes that was revealed to be potentially downstream of Dmrt2a, is related to cardiovascular development (Additional file 4: Figure S4a, b).

We proceeded to validate the microarray data by qPCR. All the selected genes were independently validated, except for ctslb in Region 1 (Fig. 10a, a’ and Additional file 5: Figure S5a, a’).

**Validation of six genes downstream of Dmrt2a: foxj1b, pxdc1b, cxcl12b, etv2, foxc1b and cyp1a**

To ensure that the selected genes were not a result of dmrt2a misexpression, we determined their level of expression in a knockdown context, using the previously validated dmrt2a-MO. In this combinatorial approach, only the genes down-regulated in the gain-of-function and up-regulated in the loss-of-function, or vice-versa, would be considered to be truly downstream of Dmrt2a activity.

In embryos injected with control-MO or dmrt2a-MO, we microdissected Region 1 and Region 2 (Fig. 9b, c) and did qPCR of the previously validated genes (Fig. 10b, b’ and Additional file 5: Figure S5b, b’).

Using this strategy, we were able to validate five genes: foxj1b, pxdc1b, cxcl12b, etv2 (Region 1) and cyp1a (Region 2) (Fig. 10a-b; for an illustration of the expression pattern of these genes see Additional file 6: Figure S6A1-J2, and for dmrt2a expression pattern see Additional file 7: Figure S7A1-B2). To ensure that the change of the expression levels of the five selected genes was not due to dmrt2a-MO off-target effects, we injected this MO in the mutant background and compared it with wildtype embryos. We analysed these embryos using in situ hybridisation and showed that these genes are specifically affected by dmrt2a-MO (Additional file 7: Figure S7C1-C3 and Additional file 8: Figure S8A1-E3). We also observed that similarly to...
what happens in \( \text{dmrt2a} \) mutants, in morphant embryos, \( \text{dmrt2a} \) transcripts are also up-regulated (Figs. 7 and 10b, Additional file 5: Figure S5b and Additional file 7: Figure S7A1-A2).

As Etv2 is a transcription factor with a well-described interaction with the Foxc proteins [20, 21], we verified the presence or absence of Foxc proteins in the microarray data. We found that the zebrafish homolog genes of the mouse \( \text{Foxc1} \) – \( \text{foxc1a} \) and \( \text{foxc1b} \) – were present in the microarray data but with low fold changes (FC = −1.04, \( P = 0.219 \), FDR = 0.636; FC = −1.3, \( P = 0.00134 \), FDR = 0.103, respectively) (Additional file 3: Figure S3a). While \( \text{foxc1a} \) could not be validated by the loss-of-function approach, we verified that \( \text{foxc1b} \) was indeed down-regulated after \( \text{dmrt2a} \) overexpression and up-regulated after \( \text{dmrt2a} \) loss-of-function (Fig. 10c, c’, for an illustration of the expression pattern of \( \text{foxc1b} \) see Additional file 6: Figure S6K1-L2). To ensure that the change of the expression levels of \( \text{foxc1b} \) was not due to \( \text{dmrt2a} \)-MO off-target effects, we injected this MO in the mutant background, as before. We analysed these embryos using in situ hybridisation and showed that \( \text{foxc1b} \) is specifically affected by \( \text{dmrt2a} \)-MO (Additional file 8: Figure S8F1-F3’).

In this work, we identified and validated six genes downstream of Dmrt2a during early development: \( \text{foxj1b} \), \( \text{pxdc1b} \), \( \text{cxcl12b} \), \( \text{etv2} \), \( \text{foxc1b} \) and \( \text{cyp1a} \). The relationship between them and Dmrt2a, and their contribution to the different Dmrt2a functions, will be the subject of future studies.

**Discussion**

In order to establish the mechanism of action of Dmrt2a, one needs to identify its downstream genes. In this work, we generated and validated several genetic tools that allowed us to identify six genes downstream of
Dmrt2a during early embryonic development: foxj1b, pxdc1b, cxcl12b, etv2, foxc1b and cyp1a.

To develop one of our genetic tools – stable dmrt2a mutant lines – we used the TALEN technology. However, when considering the different dmrt2a mutant lines we generated, we were surprised to detect only a very weak or negligible phenotype, when compared to the previously published morpholino data [3, 5, 8]. The robustness of dmrt2a knockdown phenotype had been previously established in two independent studies [3, 5], especially in the work of Matsui et al. [5], and its specificity was further confirmed with our work.

The weak phenotype obtained could not be due to the maternal contribution of dmrt2a transcripts because we always used an incross between homozygous mutant fish. The presence of alternative ATGs could be an issue, as discussed before, mainly in the case of dmrt2b mutant. Another possibility for the weak mutant phenotype is the presence of compensatory gene(s). Our data indicate that dmrt2b does not seem to compensate for the lack of dmrt2a, supporting the findings of Liu et al. [8]. Therefore, to uncover if a compensatory mechanism is taking place, a genome wide approach could be performed comparing mutants and morphants.

To prove the absence of mutant proteins we need specific antibodies that are not available. Thus, in an attempt to understand the outcome of our mutations, we evaluated dmrt2a and dmrt2b transcripts in the different mutant backgrounds. In theory, if the transcripts are not functional they should be degraded. This hypothesis should have been true at least in the case of dmrt2b mutant, due to non-sense mediated decay. However, we observed that dmrt2a and dmrt2b transcripts are up-regulated in the different mutants evaluated. This suggests that, in the absence of a completely functional transcript/protein, the stimulus to constantly produce more transcript remains, further suggesting that Dmrt2a and Dmrt2b proteins, if produced, should not be completely functional. This does not exclude the possibility of degradation of the mutant transcripts, only that the rate of transcript degradation is slower than the rate of production.

Regarding future approaches to this problem, an effort should be placed in the identification of dmrt2a and dmrt2b promoter regions, in the generation of bona fide null mutants and in the production of specific antibodies.

As we have no certainty that dmrt2a mutants are nulls, we decided against using these lines in the microarray approach. Yet, we were able to take advantage of them to validate dmrt2a-MO, confirming its specificity. Therefore, we performed the microarray with Tg(hsp70:HA-dmrt2a) and validated its data using dmrt2a-MO.
From the list of microarray validated genes, foxj1b [19] is a strong candidate to mediate the left-right asymmetry phenotypes of Dmrt2a, which were previously described in gain and loss-of-function approaches.

With our timely controlled dmrt2a overexpression experiments we identified a new phenotype in somite border formation, which recalls the Dmrt2 mouse mutant phenotype [9]. The defects observed in the mouse mutants were associated with a strong reduction of the levels of LAMININ-1, an extracellular matrix protein [9]. While in our pool of six validated genes, we did not identify classic extracellular matrix related genes, we found an interesting candidate, pxdc1b. Pxdc1b belongs to a family related to membrane attachment to organelles of the endocytic and secretory systems via binding of phosphoinositide lipids [22]. As extracellular matrix proteins are continuously being secreted [23], pxdc1b could establish a link between Dmrt2a, extracellular matrix deposition and somite border defects.

Additionally, in our microarray, we identified cyp1a as a gene downstream of Dmrt2a. This cytochrome was one of the few genes up-regulated after dmrt2a overexpression and the only one that was validated by the loss-of-function approach. Also, cyp1a is the only validated gene from the microdissected Region 2. However, Cyp1a physiological function in the embryo remains unknown therefore, its interaction with Dmrt2a will require further analysis.

Interestingly, we validated three genes related to vascular development: cxcl12b, etv2 and foxc1b. Although we lack functional data to explain the relationship between Dmrt2a and these genes, when considering dmrt2a expression pattern within the somite, we can contemplate the hypothesis that Dmrt2a is affecting the small population of somite-derived endothelial cells [24, 25].

Cxcl12b is expressed in a subset of the myotome, named endotome, which will colonise the dorsal aorta and will contribute to endothelial and vascular associated cells in zebrafish [25]. As dmrt2a is also expressed in the myotome [2], and as it acts as a transcriptional repressor, we can speculate that it could restrict cxcl12b endotome expression domain.

etv2-expressing angioblasts (endothelial cells precursors) migrate from the lateral plate mesoderm,
intersomitically, towards the midline, where they contribute to the dorsal aorta and posterior cardinal vein [26]. Therefore, a possible cell non-autonomous interaction between Dmrt2a and etv2 could occur at somite territory.

Foxc proteins have been previously described to interact with etv2 [21] thus, it would be worthwhile to analyse if, the somite-expressed gene foxc1b, can cooperate with etv2 downstream of Dmrt2a. Moreover in mouse, CXCL12 receptor – CXCR4 – is transcriptionally activated by FOXC1 and FOXC2 in endothelial cells [27]. Although in zebrafish, a link between Cxcl12 signalling and Foxc transcription factors has not been established, it would be interesting to evaluate if this interaction could occur.

Conclusions
In this study, we generated and characterised an inducible dmrt2a transgenic line and mutant lines for dmrt2a and dmrt2b. Using our transgenic line we described for the first time dmrt2a overexpression phenotype. We confirmed that the mild phenotype present in dmrt2a mutant lines is not due to a compensatory mechanism mediated by dmrt2b. Additionally, we took advantage of one of our dmrt2a mutant lines to confirm dmrt2a-MO specificity, validating this tool.

Using our tools we performed and validated a microarray experiment that allowed the identification of six genes downstream of Dmrt2a: foxj1b, pxdc1b, cxcl12b, etv2, foxc1b and cyp1a.

The identification of these six genes will contribute to the understanding of Dmrt2a mechanism of action during zebrafish early development.

In which embryonic domains is Dmrt2a controlling these genes, if their interaction with Dmrt2a is direct or not and, how can these genes contribute to the previously described Dmrt2a phenotypes, will be addressed in future studies.

Methods
Zebrafish lines maintenance and husbandry
All zebrafish (Danio rerio) lines used had Tubingen background and were maintained at 28 °C on a 14-h light/10-h dark cycle. The wildtype fish used to generate the transgenic and mutant lines were obtained from Zebrafish International Resource Center (ZIRC). Adult zebrafish were only used as breeders. The embryos when collected were kept in an incubator at 28 °C, under the same light/dark cycle, until the appropriate stage of development [11].

When growing a new fish line, embryos were bleached before reaching 24 hpf, as described [28]. After 5 days in the incubator at 28 °C, 40–50 larvae were transferred to tanks of 3 l, where they grew during 1 month. The number of juvenile fish per litre decreased gradually until reaching adulthood. Adult breeding zebrafish (3 months old) were maintained at a density of five fish per litre.

Zebrafish were raised and maintained in a six-rack recirculating system from Tecniplast supplied by a reverse osmosis unit. Conductivity was maintained at 800 µS with ocean salt (Aquarium Systems) and pH was maintained at 7.0, and adjusted with sodium bicarbonate (Sigma-Aldrich). Fish were fed with ZEBRAFEED (Sparos, Portugal) < 100 µm (larvae), between 100 and 200 µm (juvenile fish) and between 200 and 400 µm (adults). Additionally, adult fish ate a meal of decapsulated Artemia (ZM Systems) per day.

All experiments were performed with embryos obtained from an outcross of homozygous progenitors, in the case of transgenic fish, and from an incross of two homozygous progenitors, in the case of mutant fish.

Transgenic line generation
To generate the Tg(hsp70:HA-dmrt2a), dmrt2a cDNA with an N-terminal HA-tag was placed downstream of the hsp70 heat-shock promoter in the pT2 vector (UAS-hsp70 promoter-polyA-γ-crystallin promoter-CFP). dmrt2a total RNA was extracted using TRIzol (Thermo Fisher), and cDNA was synthesised using the M-MLV Reverse Transcriptase Kit (Thermo Fisher). The transgenic construct was generated using a set of PCR reactions: (1) dmrt2a sequence was amplified using the pF-dmrt2a-HA-Nter (5′-CCAGACTACGCTTCCCTTATGACGGATCTGTCCGGCACGGAG-3′) to add the C-terminal of the HA sequence to the N-terminal of dmrt2a and a pR-dmrt2a-Stul (5′-AGGGGAAAATTACGATTTCCGATTTAAGAAACGCC-3′) to add a Stul restriction site to the C-terminal of dmrt2a and, (2) using the primer pF-HA-K-ClaI (5′-CCATCGATGGCCACCATGGCTTCATATCCTTACGATG-3′) we generated the full-length HA sequence, added the Kozak sequence and the ClaI restriction site. This construct was cloned into the pGEM-T Easy Vector (Promega) and screened using the blue-white screening technique. Next, this construct and the pT2 vector were digested with ClaI and Stul (New England Biolabs) and gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega). A ligation reaction between the ClaI-Kozak-HA-dmrt2a-Stul fragment and the pT2 vector was performed using the T4 ligase (Promega). The positive colonies were selected and the DNA purified through a Genopure Plasmid Midi Kit (Roche). 20 pg (14 ng·µL⁻¹) of pT2 plasmid containing the hsp70:HA-dmrt2a insert were injected into one-cell stage zebrafish embryos together with 7 pg (5 ng·µL⁻¹) of transposase mRNA.

Southern blot
Southern blot analysis was performed as described [29]. Briefly, F1 heterozygous adult transgenic fish were
anaesthetised using Tricaine (Sigma-Aldrich). The tail fin was cut, and genomic DNA was extracted. About 10 μg of DNA were digested with Stul (New England Biolabs), and the DNA fragments were separated by gel electrophoresis, at 30 V overnight. Afterwards, the gel was stained and washed, and the DNA transferred to a nylon membrane positively charged (Roche). To cross-link the DNA, the membrane was irradiated using UV light (λ = 254 nm). The pre-hybridisation and hybridisation were performed using the ULTRAhyb buffer (Ambion). The radiolabelled probe was added at 10⁹ cpm.mL⁻¹ of ULTRAhyb, and the hybridisation was carried out at 42 °C for 16–24 h. The γ-crystallin probe was amplified from the pT2 vector using the set of primers, pFCrisSB1 (5’-ATACGACACTGCATGGGATC ACCTGAAAG-3’) and pRCrisSB1 (5’-CTTATCCCA AAGAGTTATCCAGCATTCC-3’), which generated a fragment of 561 bp. The PCR product was gel purified using the Wizard SV Gel and PCR Clean-Up System (Promega). The DNA was labelled with 3²P-dATP (PerkinElmer) using the Random Primer Labelling Kit (Stratagene). The signal was detected using the Phosphorimager Cassette ( Molecular Dynamics) and analysed using the Typhoon System Model 9210 (GE Healthcare Life Sciences).

Heat-shock experiments
In all heat-shock experiments, embryos were raised at 28 °C. At bud-stage, embryos were placed in embryo medium (5.02 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂,2H₂O, 0.33 mM MgSO₄,7H₂O, 10⁻⁵% methylene blue) pre-warmed at 39 °C, and heat-shocked for 30 min at the same temperature. Embryos were then collected and maintained at 28 °C until the appropriate stage of development. Tg(hsp70:HA-dmrt2a) and control embryos were heat-shocked with the same procedure.

Genome editing
TALEN were designed using TAL Effector-Nucleotide Targeter (TALE-NT) 2.0 web based tools [30] together with the R-based tool developed by Jorge Velez (NICHD). The selected genomic regions for dmrt2a and dmrt2b were introduced in TALEN Targeter. Custom Spacer/Repeat Variable Diresidue (RVD) Lengths were introduced in TALEN Targeter. Custom type counterparts, to ensure a controlled procedure.

For dmrt2a, we designed one TALEN pair with a spacer containing the ATG start codon, Pair-1 (P₁) (CTTT GCTTTCACCGGCGCCAAATGACGGATCTGTCCG G start codon and the DM GCACGGAAGTTTGAGA), and another TALEN pair further downstream, Pair-2 (P₂) (TCAGCCGCGTGTTCACGGGCCCCCGGAGAGGACGA CAGGGGTTCAAAGACGCGACGACAAA). For dmrt2b, we designed a TALEN pair targeting the region between the ATG start codon and the DM domain, Pair-3 (P₃) (TGCGACCACCTAGATGCTGATAGAACGCAGGAT GGGTTGGAGACGCATGGCGCCGAGCGGGAGA). TALEN binding sites are in bold caps, and the spacer region is underlined. Paired Target Finder was used to check for off-targets, the Score Cut-off chosen was 3.0.

To generate the single dmrt2a mutants (dmrt2aΔ100 and dmrt2aΔ14), 200 pg of P₁ and 200 pg of P₂ RNA were co-injected into one-cell stage embryos. To generate the single dmrt2b mutants, 250 pg of P₃ RNA was injected into one-cell stage embryos. RNA was synthesised using mMESSAGE mMACHINE SP6 Transcription Kit (Thermo Fisher). To generate the double mutants, dmrt2aΔ100−/− was crossed with dmrt2b−/− and dmrt2aΔ14−/− was crossed with dmrt2b−/−.

To screen the F0 mosaics and the following generations of dmrt2b single and double mutants, a High Resolution Melting screening method was developed using Corbett Rotorgene 6000 (Corbett Life Science) and Power SYBR Green PCR Master Mix (Thermo Fisher). The primers used for this method were: pF-HRMdmrt2b (5’-CA CAGTAGATCGACCCAC-3’) and pHRMdmrt2b (5’-C TCACTCCGTGCCCATGCC-3’). To screen dmrt2aΔ100 and dmrt2aΔ14 single and double mutants, a PCR followed by gel electrophoresis was used with the following primers: pFdmrt2a (5’-GACACGTTACATGCAGGAAAACA-3’) and pRdmrt2a (5’-GCGGTCAAGATTGTCGTGCTG-3’).

Morpholino microinjection
A dmrt2a antisense morpholino targeting the ATG region (5’-AGATCCGTCAATTTTCTGCGCCTGTA-3’) [3, 5] and a standard control morpholino (5’-CCTTCTACCA TACATTACAAATTATA-3’) obtained from Gene Tools, were injected at 1.5 ng/embryo, into one-cell stage embryos. In the morpholino validation experiments, we injected dmrt2a morpholino in the mutant background, in the same days and using the same settings as in the wild-type counterparts, to ensure a controlled procedure.

In situ hybridisation and immunohistochemistry
Single whole-mount in situ hybridisation was performed as described [31]. Embryos were photographed with a LEICA Z6 APO stereoscope coupled to a LEICA DFC490 camera.

Digoxigenin-labelled antisense RNA probes were synthesised from DNA templates of deltac [32], her1 [33], her7 [34], mespb [35], pcdh8 [36], myod1 [37], cb1045 [38], spaw [39], pitx2 [40], myl7 [41], excl12b [25], etv2 [42], foxj1b [19], foxl1b [43], dmrt2a [4], cyp1a, which was cloned in pGEM-T Easy (Promega) using the pF-cyp1a (5’-ATGGCTCTGACTTATTCTCTTCAAATATTG GG-3’) and pR-cyp1a (5’-CTAGAAAGGGACTTTGCGG
overnight with rabbit anti-HA antibody (1:1000, Y-
For HA-tag detection, the membranes were incubated
The following procedure was performed as described [45].
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bated with rat anti-HA antibody (1:200, 3F10, Roche
after heat-shock in 4% paraformaldehyde overnight, incu-
the appropriate stage, using TRIzol (Thermo Fisher) and
Total RNA was extracted from zebrafish embryos with
Quantitative RT-PCR
were microdissected as described [46]. Tissues from
heat-shock, Tg(hsp70:HA-
Between the period of 2 h and 2 h 30 min after
Microarray
were analysed with Affymetrix Zebrafish Gene 1.1 ST
ware, Affymetrix Transcriptome Analysis Console Soft-
ture (Thermo Fisher). The raw data (CEL files)
tistical analysis performed in the microarray experi-
Statistical analysis
All experiments were performed with at least three bi-
one-way ANOVA and in qPCR experiments was a two-tailed t-test. P values of less than 0.05 were considered significant.

Additional files

Additional file 1: Figure S1. TALEN pairs. a Alignment between TALEN
P1 and dmrt2a sequence. b Alignment between TALEN P2 and dmrt2a sequence. c Alignment between TALEN P3 and dmrt2b sequence. The
start codon is underlined, and the spacer region is in lowercase. Each
colour represents a different Repeat Variable Driesiude (RVD). (TIF 3783 kb)

Additional file 2: Figure S2. HA-Dmrt2a dynamics after heat-shock.
Immunostaining showing the expression of the HA-Dmrt2a fusion protein
at different time-points post-heat-shock. The HA-Dmrt2a protein is depicted
in red, and the nucleus is depicted in blue. Min: minutes. Scale bar: 10
μm. (TIF 16094 kb)

Additional file 3: Figure S3. Microarray data. a List of all the genes
obtained in the microarray with FC ≥ 2 or FC ≤ −2, P < 0.05 and FDR < 0.05,
together with dmrt2a, foxc1a and foxc1b in Region 1. (FC = 1.3, P = 0.000416,
FDR = 0.0677; FC = −1.04, P = 0.219, FDR = 0.636; FC = −1.3, P = 0.00134,
CATCGGCGATTTTTGAGGGCA-3′) and pxdc1b, which was cloned in
gEM-T Easy (Promega) using the pF-pxdc1b (5′-ATGG
CATGGCGATTTTTGAGGGCA-3′) and pR-pxdc1b
(5′-AAGTCAGTTTCAAAGGAACCAGA-3′).

For HA immunohistochemistry, the embryos were fixed
after heat-shock in 4% paraformaldehyde overnight, incub-
ated with rat anti-HA antibody (1:200, 3F10, Roche
#11867423001) followed by anti-rat Alexa Fluor 594
(1:500, Thermo Fisher #A11007). F-actin and nuclei were detected with Alexa Fluor 488-Phalloidin (1:400, Thermo
Fisher #A12379) and DAPI (10 μL.mL
−1
), respectively. Embryos were photographed with a Zeiss LSM 510 Meta.

Western blot
Embryos were collected 1 h 30 min and 2 h 30 min after heat-shock, treated as described [44] and kept at – 70 °C. The following procedure was performed as described [45]. For HA-tag detection, the membranes were incubated overnight with rabbit anti-HA antibody (1:1000, Y-
sc-805, Santa Cruz). For beta-actin, we used a rabbit anti-beta-actin (1:5000, ab68227, Abcam). As secondary antibody, we used an HRP-conjugated mouse anti-rabbit IgG antibody (1:10000, sc-2357, Santa Cruz).

Microarray
Between the period of 2 h and 2 h 30 min after heat-shock, Tg(hsp70:HA-dmrt2a) and wildtype embryos were microdissected as described [46]. Tissues from Region 1 and Region 2 (Fig. 9a, c) were kept in TRIzol (Thermo Fisher) at – 70 °C. We obtained a pool of 100 pieces from Region 1 and 100 pieces from Region 2, from each condition, per replicate. Three biological replicates for each condition were used. Total RNA was extracted using TRIzol (Thermo Fisher) and further purified with RNA Clean & Concentrator-5 kit (Zymo Research). RNA quality was assessed with an Agilent 2100 Bioanalyzer (Agilent Genomics), and all samples had a RIN value between 8.60 and 9.30. All samples were analysed with Affymetrix Zebrafish Gene 1.1 ST Array Strip (Thermo Fisher). The raw data (CEL files) were analysed with Affymetrix Expression Console Software, Affymetrix Transcriptome Analysis Console Software (Thermo Fisher) and R. Statistical analysis was performed using one-way ANOVA and False Discovery Rate (FDR) correction. Only genes with a fold change (FC) higher than 2 or lower than – 2, with P < 0.05 and FDR < 0.05, were considered with the exception of foxc1b with a FC = −1.3, P = 0.00134 and FDR = 0.103. Gene Ontology (GO) analysis was performed using AmiGO [47] with Bonferroni correction.

Quantitative RT-PCR
Total RNA was extracted from zebrafish embryos with the appropriate stage, using TRIzol (Thermo Fisher) and further purified using the Direct-zol RNA Miniprep Kit (Zymo Research). For the evaluation of dmrt2a transcript throughout development, we used n = 30 wildtype embryos, per replicate. For the gain-of-function experiment, we collected embryos from the Tg(hsp70:HA-dmrt2a) line and wildtype embryos, and performed a heat-shock (Fig. 9a). All embryos were microdissected in Region 1 and Region 2 (Fig. 9a, c). From this approach, we obtained a pool of 60 pieces from Region 1 and a pool of 60 pieces from Region 2, from each condition (experimental and control), per replicate. For the loss-of-function experiment, we injected wildtype embryos with dmrt2a-MO or control-MO, and microdissected them in Region 1 and Region 2 (Fig. 9b, c). From this approach, we obtained a pool of 46 pieces from Region 1 and a pool of 46 pieces from Region 2, from each condition (experimental and control), per replicate. For the evaluation of mutant transcripts, we used the different dmrt2a and dmrt2b mutant embryos and wildtype embryos, at 24 hpf, with n = 10, per replicate. Three biological replicates were used in each experiment. cDNA was synthesised using DyNAmo cDNA Synthesis Kit (Thermo Scientific) using random hexamer primers. Quantitative PCR was performed using Corbett Rotorgene 6000 (Corbett Life Science) and Power SYBR Green PCR Master Mix (Thermo Fisher). Quantification of the relative expression was performed using the 2ΔΔCt method. We evaluated each gene on an individual basis, between experimental and control samples. Data were analysed using a two-tailed t-test. The primers used are listed in Additional file 9: Table S1.
Additional file 4: Figure S4. Gene Ontology (GO) analysis of the microarray data. a, b Most significant GO terms associated with the microarray data with FC > 2 or FC < −2, P < 0.05 and FDR < 0.05. (a) Region 1 and (b) Region 2. (TIF 1435 kb)

Additional file 5: Figure S5. qPCR validation of the microarray data in gain and loss-of-function experiments. a, a' PCR validation of selected genes from the microarray in a gain-of-function approach. (a) Region 1 and (a') Region 2. b, b' PCR validation of selected genes from the microarray in a loss-of-function approach using dmrta-MO. (b) Region 1 and (b') Region 2. *P < 0.05, **P < 0.01, ***P < 0.001; ns: not significant. P-values were generated using a two-tailed t-test. (TIF 1349 kb)

Additional file 6: Figure S6. No misexpression was found in the validated genes after dmrta gain and loss-of-function experiments. A1-L2 In situ hybridisation results depicting the six genes validated in this work. A1-B2 ev Bang expression pattern after injecting ctmO (A1-A1), dmrta-MO (A2-A2), and after heat-shock in control (B1-B1) and Tghsp70-HA-dmrta2a (B2-B2). C1-D2 foxb1 expression pattern after injecting ctmO (C1-C1), dmrta-MO (C2-C2), and after heat-shock in control (D1-D1), and Tghsp70-HA-dmrta2a (D2-D2). E1-F2 cyp1a expression pattern after injecting ctmO (E1-E1), dmrta2-MO (E2-E2), and after heat-shock in control (F1-F1) and Tghsp70-HA-dmrta2a (F2-F2). G1-H2 exol2b expression pattern after injecting ctmO (G1), dmrta-MO (G2), and after heat-shock in control (H1) and Tghsp70-HA-dmrta2a (H2). (TIF 570 kb)

Additional file 7: Table S1. Primers used in quantitative RT-PCR. (DOC 97 kb)

Abbreviations
cDNA: Complementary DNA; ctr: Control; DM: Doublesex and Mab3; DMT: Doublesex and Mab3 related transcription factors; FC: Fold change; FDR: False discovery rate; GO: Gene ontology; HA: Haemagglutinin; hpf: Hours post-fertilisation; LRO: Left-Right Organiser; MO: Morpholino; mRNA: Messenger RNA; ns: Not significant; P: TALEN pair 1; P: TALEN pair 2; P1: TALEN pair 3; RN: RNA integrity number; RVD: Repeat variable dinucleotide; TALEN: Transcription activator-like effector nucleases; UTR: Untranslated Region; WT: Wildtype

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Availability of data and materials
The datasets supporting the conclusions of this article are available in the Gene Expression Omnibus (GEO) repository, with the accession number GSE103105 (https://www.ncbi.nlm.nih.gov/geo/).

Authors’ contributions
RAP performed all the experiments, analysed the data and wrote the manuscript. JA-S designed the TALEN pairs, did the TALEN microinjections and optimised the mutant screening assays. EI conceived the plan for the transgenic line. LS conceived the project, analysed the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All the procedures performed in zebrfish were done at the Instituto de Medicina Molecular’s fish facility, licensed by the national General Directorate authority (DGAV – Direcção Geral de Alimentação e Veterinária) complying with the Directive 2010/63/EC (transposed to the Portuguese legislation by Decreto-Lei 113/2013) and following the Federation of European Laboratory Animal Science Associations (FELASA) guidelines and recommendations concerning laboratory animal welfare, scientific use and proper education/training of all personnel performing animal work. The present project was approved by the ethics committee of Centro Hospitalar Lisboa Norte and Centro Académico de Medicina de Lisboa, presided by Professor Doctor José Luís B. Duca Soares.

Competing interests
The authors declare that they have no competing interests.

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