Supporting Information

Cardiovascular effects and molecular mechanisms of bisphenol A and its metabolite MBP in zebrafish

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Table S1: The zebrafish heart and heart valve morphogenesis (valvulogenesis)

The zebrafish heart comprises a single atrium and ventricle, as opposed to two atria and a single ventricle in reptiles and amphibians, or two atria and two ventricles in birds and mammals (Stainier, 2001). Single circuit, systemic blood circulation in the zebrafish is regulated by three valves separating the sinus venosus-atrium, atrium-ventricle, ventricle-bulbus arteriosus, each of which help to minimise retrograde blood flow (Hu et al., 2000; Tessadori et al., 2012). In air breathing vertebrates there is a systemic circuit and a pulmonary (lung) circuit. Despite these gross morphological differences, the cellular and molecular architectures of the zebrafish heart, including the heart valves, are highly similar to those in mammals (Beis et al., 2005) and clearly illustrate the common evolutionary origin of these structures (Staudt and Stannier, 2012).

The bulbo-ventricular canal (BVC) and the atrio-ventricular canal (AVC), which are precursors to the BV and AV valves respectively, can be distinguished at 36 hours post fertilisation (hpf) (Mehta et al., 2008) and the sinus venosus valve is distinguishable by 48 hpf (Grimes et al., 2006). Initial valvulogenesis (formation of the endocardial rings) is completed shortly after 96 hpf (Hu et al., 2000; Grimes et al., 2006) and is described in detail for the atrio-ventricular valve (Hove et al., 2003; Bartman et al., 2004; Vermot et al., 2009; Staudt and Stainier, 2012; Chen et al., 2013), but not the bulbo-ventricular valve or sinus venosus valve. By 48 hpf, the AVC endocardium thickens to form the endocardial ring, and by 55 hpf this consists of a single\ layer of polarized cuboidal endocardial cells that stain strongly for alcama (Scherz et al., 2008). Cuboidal cell formation and alcama expression is dependent on troponin T type 2a (tnnt2a) (Bartman et al., 2004; Beis et al., 2005). The cuboidal endocardial cell layer subsequently proliferates, folds and extends into the extracellular matrix to form the superior valve leaflet by 85 hpf, and the inferior leaflet by 102 hpf (Scherz et al., 2008). The valve leaflets consist of two layers of cells, with those in the layer closest to the AVC remaining cuboidal and those in the layer closest to the myocardium developing a rounded shape (Scherz et al., 2008). Heart valve formation and remodelling (e.g. AV valve transitioning from two to four leaflets) is completed by 35 dpf (Beis et al., 2005, Sarmah et al., 2016). At the a molecular level, initial formation of the AVC coincides with localised expression of *bmp4*, versican (*cspg2*) and *tgfb* in the AVC myocardium (Walsh and Stainier, 2001; Beis et al., 2005; Chen et al., 2013) and expression of *notch1b*, calcineurin (*ppp3ca, b, c* and *ppp3r1, 2*) (Beis et al., 2005); *has2* (Hurelstone et al., 2003) and *prss23* (Chen et al., 2013) in the AVC endocardium. Key genes (e.g. *notch1b* and *bmp4*) in gene networks regulating endocardial cell proliferation and valve morphogenesis (post 48 hpf) have been linked to Wnt/β-catenin signalling (Walsh and Stainier, 2001; Hurlestone et al., 2003), TGF-β, ErbB/Neuregulin and prostaglandin signalling (Scherz et al., 2008) and to *pkd2/Hdac5/Klf* signalling (Vermot et al., 2009).

Table S2: Water quality measured during BPA and MBP exposure studies

Exposure treatment	Temperature (°C)	pH	Dissolved oxygen (%)	Ammonia (µg/L NH_3)	Hardness (mg/L $CaCO_3$)
BPA (0-5 dpf)	27.4-28.1	7.7-7.8	84-98	1.7-2.2	100-125
BPA (0-15 dpf)	26.8-27.9	7.7-7.9	79-96	1.4-3.2	100-125
MBP (0-5 dpf)	27.7-28.0	7.5-7.9	74-91	1.6-2.3	100-125
MBP (0-15 dpf)	26.7-27.9	7.4-7.8	87-93	1.5-2.9	100-125

Data represent the range in water quality measurements for solvent controls, low and high-level exposure treatments in each study.

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Table S3: Test substances and analysis

Chemical Abstracts Service (CAS) Number	Compound name	Structure
80-05-7	BPA 2,2-Bis(4-hydroxyphenyl)propane	HO, OH
13464-24-9	MBP 4-methyl-2,4-bis(4-hydroxyphenyl)pent-1-ene	HO, OH

Analytical method

Analysis of water and tissue samples was performed by Liquid Chromatography and Mass Spectrometry (LC-MS).

Chromatographic separation was achieved using a reversed-phase, 3 µm particle size, C18 Hypersil GOLD column (50 mm × 2.1 mm i.d., Thermo Scientific, San Jose CA, USA).

Both analytes were separated using a linear gradient of (A) aqueous phase and (B) organic solvent with initial conditions shown in the table below. Solvent B increased to 100% in 4.5 min and this was maintained for 1 min, before returning to the initial condition. The flow rate was 500µL/min. Temperature of autosampler was set at 8˚C, while column was kept at a room temperature.

Analyte	(A) Aqueous phase	(B) Organic phase	Initial conditions (% of B)
BPA	Water	Methanol	10
MBP	Water	Methanol	10

Mass spectrometry was performed using a TSQ Vantage triple quadrupole mass spectrometer. The mass spectrometer was equipped with a heated electrospray (HESI II) source (ThermoFisher Scientific, Hemel Hempstead, UK). The HESI probe was operating in both negative and positive mode; an ion-spray voltage of -4.0 kV for both analytes. The heated capillary temperature was set at 275 °C and the vaporizer temperature was 60 °C. Nitrogen was employed as a sheath and auxiliary gas at a pressure of 60 and 2 arbitrary units, respectively.

The argon CID gas was used at a pressure of 1.5 mTorr and the optimum collision energy (CE) for each transition was selected. Quantification of the target compounds was performed by monitoring two characteristic multiple reaction monitoring (MRM) transitions (Table below).

Analyte	Parent ion (m/z)	Product ion (m/z)	CE (eV)
BPA	227	133.1	23
		117.0	49
MBP	267.1	212.1	20
		133.1	28

Table S4: BPA and MBP concentrations in water and zebrafish larvae (5 days post fertilization)

Aqueous exposure conc. (µg/L)	Replicate aquaria	Measured aqueous conc. (µg/L)	Measured whole body conc. # (ng/g)	Bio-concentration factor ¥ $BCF_{whole\ body}$	Measured heart conc. § (ng/g)	Bio-concentration factor ¥ BCF_{heart}
BPA exposure study						
Solvent Control	1	0	< LOD	0	0	0
	2	0	< LOD	0	0	0
	3	0	< LOD	0	0	0
100	1	115	270	2.6	-	-
	2	108	257	2.5	-	-
	3	128	240	2.4	-	-
1000	1	1069	3190	3.2	62	0.06
	2	1051	3660	3.7	57	0.06
	3	1042	4390	4.5	140	0.14
MBP exposure study						
Solvent Control	1	0	< LOD	0	-	-
	2	0	< LOD	0	-	-
	3	0	< LOD	0	-	-
2.5	1	2.4	-	-	-	-
	2	2.0	-	-	-	-
	3	1.8	-	-	-	-
25	1	28	705	25.2	-	-
	2	29	732	25.3	-	-
	3	27	592	21.9	-	-

Data are presented as the mean ± 95% confidence interval.

Whole body concentration was calculated based on a wet weight of 1200 µg for a single zebrafish larvae at 5 days post fertilization (dpf) (Hu et al., 2000).

§ Heart concentration was calculated based on a ventricle weight of 10% of the whole body weight at 5 dpf (Hu et al., 2000).

¥ Bio-concentration factor (BCF) was calculated as measured tissue concentration / measured aqueous exposure concentration.

Aqueous exposure concentrations were relatively stable of over time. During the longest period between the static renewal of exposure solutions (i.e. 0-5 days), maximum reductions in aqueous exposure concentrations were: 34% for BPA (from 108% to 74% of nominal for the 100 µg/L exposure); 29% for MBP (from 96% to 67% of nominal for the 2.5 µg/L exposure).

Table S5: Relative mean fluorescence intensity induced by BPA and MBP in the heart valves

	5 dpf		15 dpf	
BPA exposure (µg/L)	100	1000	100	1000
Heart valve fluorescence	2.2 ± 0.3	31 ± 2	15.3 ± 1.1	26.9 ± 0.2
MBP exposure (µg/L)	2.5	25	2.5	25
Heart valve fluorescence	34 ± 3	54 ± 5	3.0 ± 0.4	14.6 ± 1.5

Relative mean fluorescence intensity of the ERE:GFP reporter was quantified relative to the solvent control at 5 and 15 days post fertilization (dpf). Data are presented as the mean ± 95% confidence interval.

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Table S6: Effects of BPA and MBP exposure on cardiovascular function in 15 dpf zebrafish larvae

Results

Exposure Treatment	Heart beat rate (bpm)	Blood flow (nL/s)
BPA Exposure		
0	241 ± 4	2.01 ± 0.06
100	251 ± 2	2.16 ± 0.08
1000	250 ± 4	2.15 ± 0.08
MBP Exposure		
0	249 ± 7	1.95 ± 0.06
2.5	243 ± 4	1.69 ± 0.08
25	218 ± 4	1.55 ± 0.07

Data are presented as the mean ± 95% confidence interval.

MANOVA analysis with tank as a random effect

LME model fit with aquarium as a random effect

MBP Treatment 1 = control, Treatment 2 = Low (2.5 µg/L), Treatment 3 = High (25 µg/L)

Response = 'Blood flow' AND 'Heart beat rate'

```
model = lme(cbind(Blood.flow, Heart.beat.rate) ~ Treatment, random=~1|Aquarium, data = dat)

      AIC      BIC    logLik
  71.15566 77.63485 -30.57783……..etc.

             Df  Pillai     approx F  num Df den Df  Pr(>F)
Treatment  2   0.36586   3.0225      4         54       0.02539 *
Residuals 27
```

Response = 'Blood flow'

```
model = lme(Blood.flow ~ Treatment, random=~1|Tank1, data=dat)

      AIC      BIC    logLik
  71.15566 77.63485 -30.57783

Random effects: (Intercept).  Residual StdDev:   0.2323506 0.6288888

Fixed effects: Blood.flow ~ Treatment
                 Value       Std.Error   DF   t-value      p-value
(Intercept)      3.916218 0.2349573 17    16.667790  0.0000
Treatment2  -0.540965 0.3239045 10     -1.670138  0.1258
```

Treatment3 -0.831637 0.3296337 10 -2.522912 **0.0302**
Correlation: (Intr) Trtmn2; Treatment2 -0.725; Treatment3 -0.713 0.517

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-1.8354989	-0.6211684	-0.1157832	0.6755835	1.9765751

Number of Observations: 30
Number of Groups: 13

Response = 'Heart beat'
model = lme(Heart.beat.rate ~ Treatment, random=~1|Tank1, data=dat)

AIC	BIC	logLik
263.076	269.5552	-126.538

Random effects: (Intercept). Residual StdDev: 8.827086 21.79687

Fixed effects: Heart.beat.rate ~ Treatment

	Value	Std.Error	DF	t-value	p-value
(Intercept)	249.72992	8.308052	17	30.058780	0.000
Treatment2	-7.10923	11.491302	10	-0.618661	0.550
Treatment3	-31.34922	11.686427	10	-2.682532	**0.023**

Correlation: (Intr) Trtmn2; Treatment2 -0.723; Treatment3 -0.711 0.514

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-2.4816596	-0.5578130	0.1658430	0.6262645	1.3984753

Number of Observations: 30
Number of Groups: 13

Table S7: Effects of BPA and MBP exposure on specific growth rate (SGR) and critical swimming speed (U_{critb}) in 15 dpf zebrafish larvae

Results

Exposure Treatment	SGR (% standard body length per day)	U_{critb} (body lengths/sec)
BPA Exposure		
0	1.37 ± 0.10	12.72 ± 0.23
100	1.32 ± 0.07	12.28 ± 0.20
1000	1.44 ± 0.06	11.82 ± 0.19
MBP Exposure		
0	1.36 ± 0.15	12.61 ± 0.44
2.5	0.96 ± 0.11	11.70 ± 0.43
25	1.06 ± 0.07	10.73 ± 0.40

Data are presented as the mean ± 95% confidence interval.

LME model fit with aquarium as a random effect

MBP Treatment 1 = control, Treatment 2 = Low (2.5 µg/L), Treatment 3 = High (25 µg/L)

Response = 'Ucritb'

```
model = lme(Ucritb ~ Treatment, random=~1|Aquarium, data=dat)
     AIC      BIC    logLik
  255.7234 265.1825 -122.8617
```

Random effects: Tank (Intercept) Residual StdDev: 0.4352544 2.694444

Fixed effects: Ucritb ~ Treatment

```
               Value      Std.Error  DF  t-value     p-value
(Intercept)   10.992396 0.5900421 36   18.629851  0.0000
Treatment2   -0.819231 0.9344436 13   -0.876705  0.3966
Treatment3   -1.667611 0.9514960 13   -1.752620  0.1032
Correlation:  (Intr) Trtmn2: Treatment2 -0.631; Treatment3 -0.620  0.392

Standardized Within-Group Residuals:
     Min        Q1        Med        Q3        Max
-2.3945383 -0.5321397 -0.1088102  0.5557497  2.3296676
Number of Observations: 52
Number of Groups: 16
```

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Response = 'Specific Growth Rate'

model = lme(SGR ~ Treatment, random=~1|Aquarium, data=log.dat)

Linear mixed-effects model fit by REML

AIC	BIC	logLik
26.83093	30.37118	-8.415466

Random effects: Aquarium (Intercept) Residual

StdDev: 0.3319166 0.1244687

Fixed effects: SGR ~ Treatment

	Value	Std.Error	DF	t-value	p-value
(Intercept)	1.1797476	0.1447187	15	8.152003	0.0000
Treatment2	-0.3403799	0.2046632	15	-1.663122	0.1170
Treatment3	-0.2560214	0.2046632	15	-1.250940	0.2301

Correlation: (Intr) Trtmn2: Treatment2 -0.707; Treatment3 -0.707 0.500

Standardized Within-Group Residuals:

Min	Q1	Med	Q3	Max
-0.5324397885	-0.2427571025	0.0007605993	0.2696808931	0.6679405905

Number of Observations: 18

Number of Groups: 18

Table S8: Differentially expressed genes in BPA and MBP exposure treatments

In the low-level BPA exposure (100 µg/L) 131 genes were differentially expressed at 5 dpf and only 1 gene apolipoprotein Da, duplicate 2 (*apoda.2* - associated with GO:0006810 ~transport) at 15 dpf (SI Table S8 .xls). At 5 dpf there was significant enrichment of genes / ontologies associated with transport: GO:0006810 ~transport, GO:0006811 ~ion transport, GO:0055085 ~transmembrane transport, GO:0035879 ~plasma membrane lactate transport. Cell signalling pathways were also enriched GO:0007219 ~Notch signalling pathway, dre04630:Jak-STAT signalling pathway, dre04060:Cytokine-cytokine receptor interaction, dre04080:Neuroactive ligand-receptor interaction (SI Table S9a .xls).

In the high-level BPA (1000 µg/L) 371 genes were differentially expressed at 5 dpf: 62 of these genes were consistent with the low-level BPA exposure treatment at 5 dpf; 5 genes were consistent with the high-level BPA exposure treatment at 15 dpf - for which there was a total of 32 differentially expressed genes (SI Table S8 .xls). There were three distinct gene groups at 5 dpf with significantly enriched ontologies for i) 'Cellular and extracellular matrix interactions' including via KEGG pathways dre04510:Focal adhesion, dre04512:ECM-receptor interaction, and the following processes GO:0005509 ~calcium ion binding, GO:0003171 ~atrioventricular valve development, GO:0060347 ~heart trabecula formation); ii) 'Transcriptional regulation' including via dre04330:Notch signalling, GO:0001947 ~heart looping, GO:0003146 ~heart jogging, GO:0002040 ~sprouting angiogenesis; iii) 'Protein metabolism' including GO:0006508 ~proteolysis, GO:0008544 ~epidermis development, GO:0030199 ~collagen fibril organization, GO:0060429 ~epithelium development (SI Table S9c .xls). At 15 dpf the most notable among the 32 differentially expressed (down-regulated) genes were: actinin alpha 3b (*actn3b*), myosin light chain, phosphorylatable, fast skeletal muscle a (*mylpfa)*, myosin, heavy polypeptide 2, fast muscle specific (*myhz2*) associated with KEGG pathways: dre04510~ Focal adhesion, dre04520 ~Adherens junction, dre04530 ~Tight junction, dre04810 ~Regulation of actin cytoskeleton. Other differentially expressed genes at 15 dpf included insulin-like growth factor 1a receptor (*igf1ra*) associated with GO:0007507 ~heart development, and troponin I type 2a (skeletal, fast), tandem duplicate 4 (*tnni2a.4*) associated with GO:0030239 ~myofibril assembly, GO:0060048 ~cardiac muscle contraction (SI Table S9c .xls).

Low-level MBP exposure (2.5 µg/L) resulted in differential expression (down-regulation) of 8 genes at 5 dpf, associated with cellular respiration: dre00010:Glycolysis/Gluconeogenesis, oxidative stress and immune response: dre00480:Glutathione metabolism, dre00590:Arachidonic acid metabolism, and also cell signalling: dre04310:Wnt signalling pathway. Only one (unannotated) gene (*si:dkey-7c18.24*) was differentially expressed at 15 dpf (SI Table 8 .xls).

High-level MBP exposure (25 µg/L) resulted in differential expression (predominantly down-regulation) of 127 genes at 5 dpf. One of these genes: elastin microfibril interfacer 3 (*emilin3*) was consistent with the low-level MBP exposure treatment at 5 dpf, and 2 genes: activated leukocyte cell adhesion molecule b (*alcamb*) and transforming growth factor, beta-induced (*tgfbi*) were consistent with that seen for the high-level BPA exposure treatment (SI Table S8 .xls). There were two distinct gene groups with significantly enriched ontologies for i) 'Cellular and extracellular matrix interactions' including via KEGG pathways dre04510:Focal adhesion, dre04512:ECM-receptor interaction, and the process GO:0060536 ~cartilage morphogenesis; ii) 'Filamentous protein synthesis and activity' including GO:0045095 ~keratin filament, GO:0005882 ~intermediate filament, GO:0005198 ~structural molecule activity (Table 9d .xls). The transcriptomic effects of high-level MBP exposure at 15 dpf could not be established due to problems encountered in sample processing (PCR amplification of libraries).

***See separate file**: ‘Supplemental Information Tables S8 to S12.xls’ for the following tables*

.xls Tables

Table S8: Differentially expressed genes in BPA and MBP exposure treatments (continued)

Table S9: Enriched GO terms and KEGG pathways in BPA and MBP exposure treatments according to DAVID's Gene Functional Classification

Table S10: Enriched Reactome pathways in BPA and MBP exposure treatments

Table S11: Enriched Transcription Factor Binding Site motifs 5 kilobytes upstream of differentially expressed genes for BPA and MBP exposure treatments

Table S12: Enriched Transcription Factor Binding Site motifs 5 kilobytes downstream of differentially expressed genes for BPA and MBP exposure treatments

Table S13: Results summary for enriched transcription factor binding site motifs for EREs

Test chemical	Exposure conc (µg/L)	Time point (dpf)	up/ down-stream	Proximal flanking regions (5 kB)									Distal flanking regions (50 kB)	
				sp1	*sp3*	*sp4*	*creb1*	*creb5*	*nfkb2*	*foxa1*	*runx1*	*esr2*	*esr1*	*esr2*
BPA	100	5	up	✓	✓	✓	✓	✓	✓	✓	✓			✓
			down	✓	✓	✓		✓			✓			✓
	1000	5	up	✓	✓	✓	✓			✓	✓	✓		✓
			down	✓	✓	✓				✓	✓			✓
	1000	15	up	✓	✓	✓	✓							
			down	✓	✓	✓								
MBP	2.5	5	up	✓	✓	✓								
			down	✓	✓					✓			✓	
	25	5	up	✓	✓	✓	✓				✓			
			down	✓	✓	✓	✓							✓

Proximal flanking regions 5 kB up- and down-stream of differentially expressed genes are generally considered to be rich in ERE binding sites and other transcription factor binding sites related to estrogen receptor (ER)-signalling including: estrogen receptors (*esr1, esr2*); specificity proteins constituting ERE tethering factors (*sp1, sp3, sp4*); pioneer factors facilitating ER binding (*foxa1, nfkb2, pbx1, runx1*); CAMP responsive element binding proteins (*creb1, creb5*).

Distal enhancer or promoter elements (up to 100 kB) are also involved in regulating the expression of many estrogen receptor target genes, often through looping or other higher order chromatin structures (reviewed in Dietz and Carroll, 2008; Liu and Cheung, 2014; Magnani and Lupien, 2014). This highlights the difficulties in pinning down the regulation of individual genes by estrogen receptors and the benefit of wider scanning of flanking regions for sets of genes to evaluate enrichment of TFBS motifs for ER-related transcription factor binding. We elected to scan 50 kB up- and down-stream of our differentially expressed genes, since the mean intergenic region in zebrafish is 97 kB, with a standard deviation of 164 kB (Hu et al., 2015). Therefore scanning ±100 kB would have a high risk of overlapping genes.

Figure S1: Nuclear Magnetic Resonance Spectrum for 4-methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene (MBP)

4-Methyl-2,4-bis(p-hydroxyphenyl)pent-1-ene (MBP) was synthesised at the University of Exeter. The final purity of MBP was 99%.

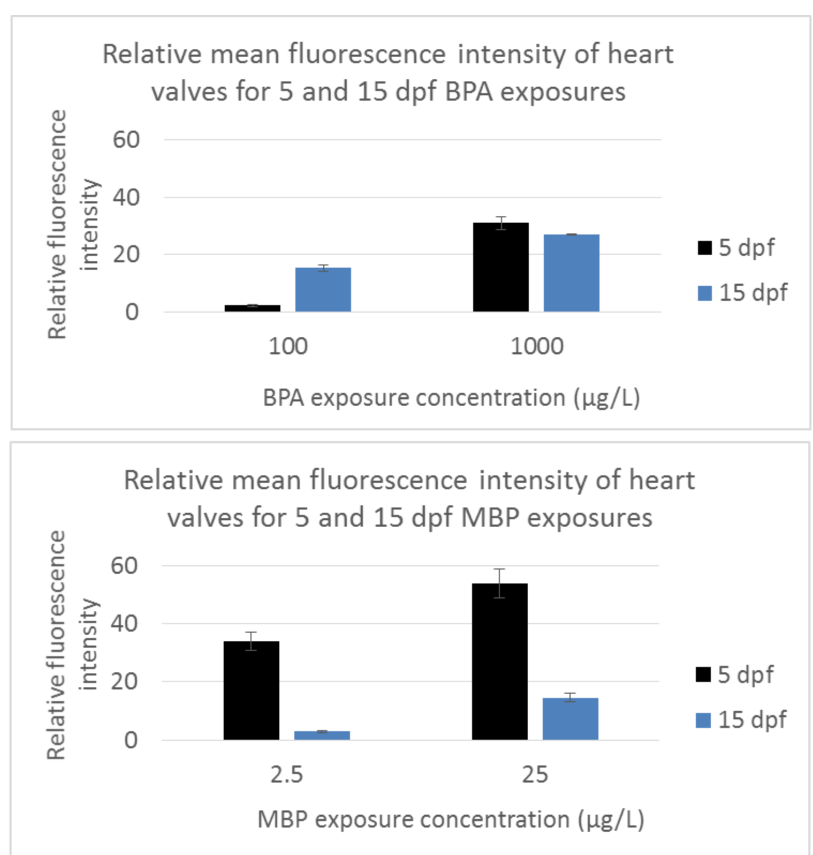


Figure S2: Relative fluorescence in the hearts of ERE-GFP transgenic zebrafish larvae at 5 days and 15 days post fertilization for exposure to BPA and MBP

Relative mean fluorescence intensity was quantified relative to the solvent controls to account for any background auto-fluorescence. Mean fluorescence was quantified from 6 individual fish taken randomly from each of 6 separate aquaria (n=6 experimental replicates) per exposure treatment. Error bars represent 95% confidence intervals.

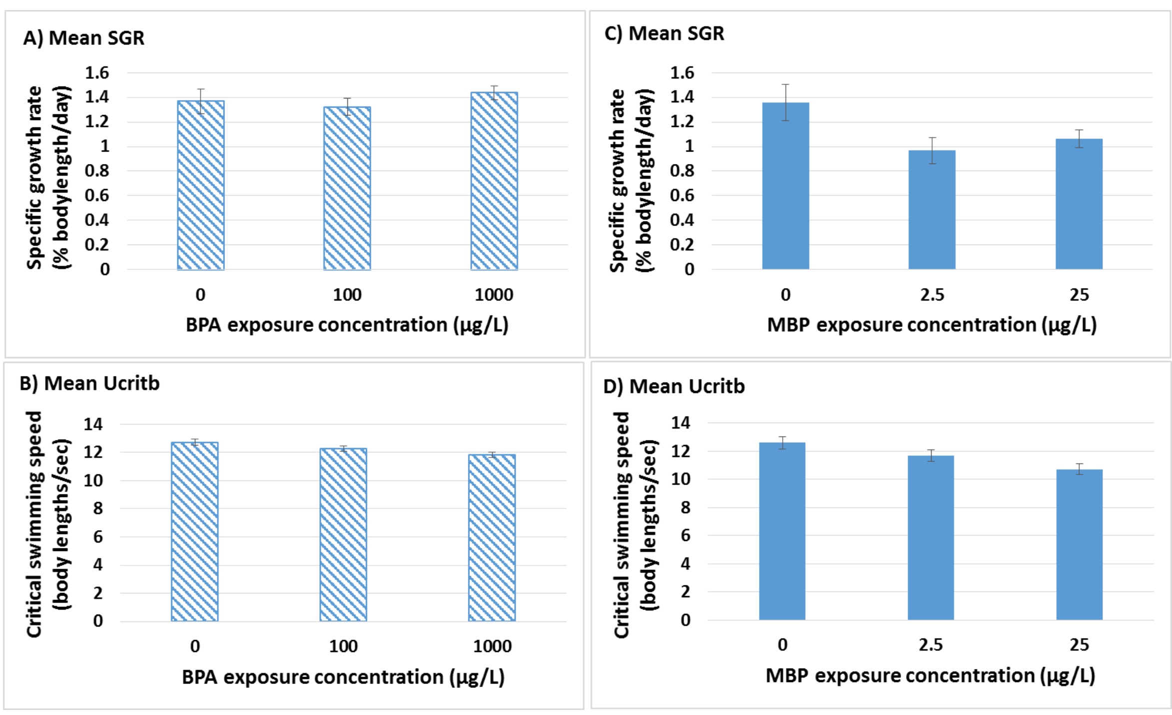


Figure S3: Effects of BPA and MBP exposure on specific growth rate (SGR) and critical swimming speed (U_{critb}) in zebrafish larvae at 15 days post fertilisation (dpf)

Hatched bar charts (A-B) represent BPA, solid bar charts (C-D) represent MBP. Bar heights represent means, error bars represent 95% confidence intervals. There were no significant effects at (p<0.05).

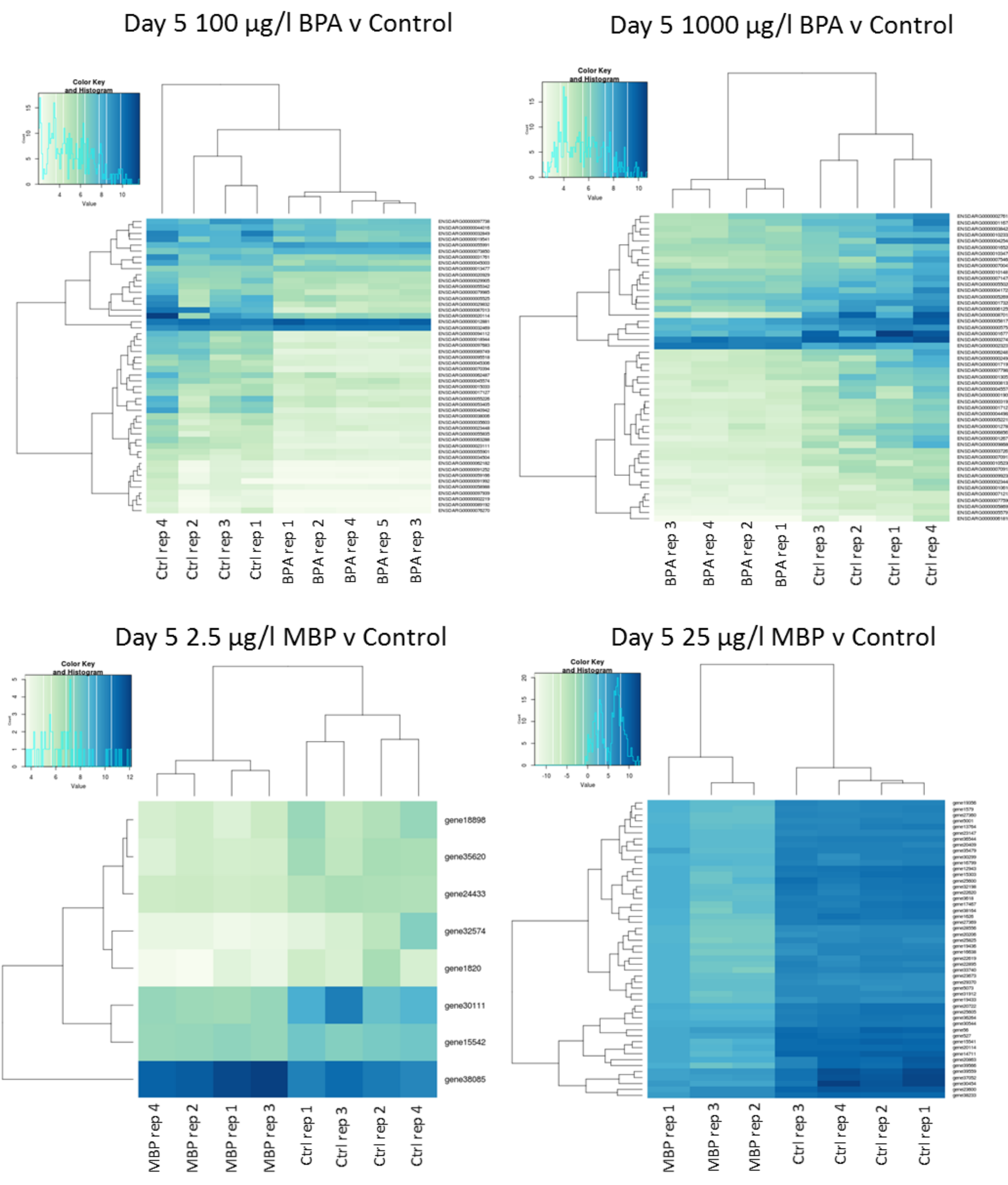


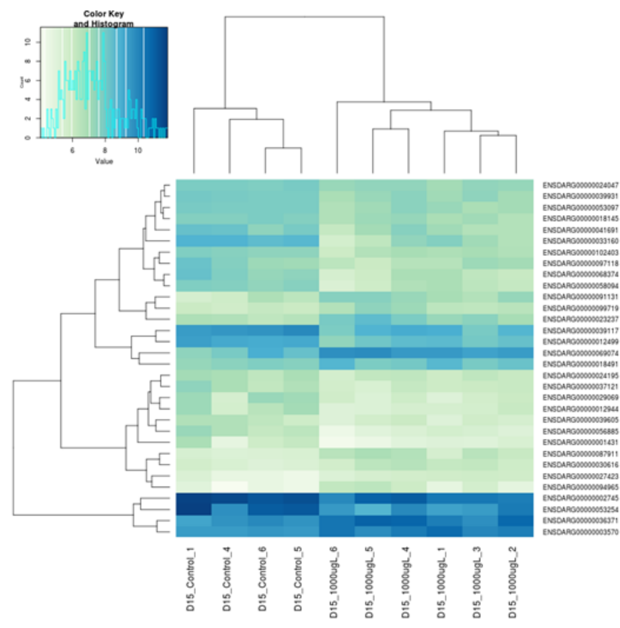
Figure S4. Differential gene expression in heart tissue sampled from BPA and MBP exposure treatments versus solvent controls in larval zebrafish at 5 days post fertilization (dpf)

Differential gene expression in embryo-larval heart tissues was assessed using DESeq2 (Love et al., 2014). An adjusted *p*-value of <0.05 was set as the false discovery rate. The most differentially expressed genes (top 50, maximum) are shown: colour gradient light-dark represents low-high gene expression. Data for experiment treatment replicates were generated from hearts pooled from ~30 individual from each of 4 separate aquaria (nominally n=4 experimental replicates) per exposure treatment.

Day 15 100 µg/l BPA v Control

Only one gene was expressed differentially between treatments (apolipoprotein Da, duplicate 2 (*apoda.2*))

Day 15 1000 µg/l BPA v Control



Day 15 2.5 µg/l MBP v Control

Only one gene was expressed differentially between treatments (apolipoprotein Da, duplicate 2 (*si:dkey-7c18.24*)

Day 15 25 µg/l MBP v Control

The transcriptomic effects of high-level MBP exposure at 15 dpf could not be established due to problems encountered in sample processing (PCR amplification).

Figure S5: Differential gene expression in heart tissue sampled from chemical exposure treatments versus solvent controls at 15 days post fertilisation (dpf)

Differential gene expression in embryo-larval heart tissues was assessed using DESeq2 (Love et al., 2014). An adjusted p-value of <0.05 was set as the false discovery rate. The most differentially expressed genes (top 50, maximum) are shown: colour gradient light-dark represents low-high gene expression. Data for experiment treatment replicates were generated from hearts pooled from ~30 individual from each of 4 separate aquaria (nominally n=4 experimental replicates) per exposure treatment.

Cardiovascular effects of BPA and MBP in zebrafish

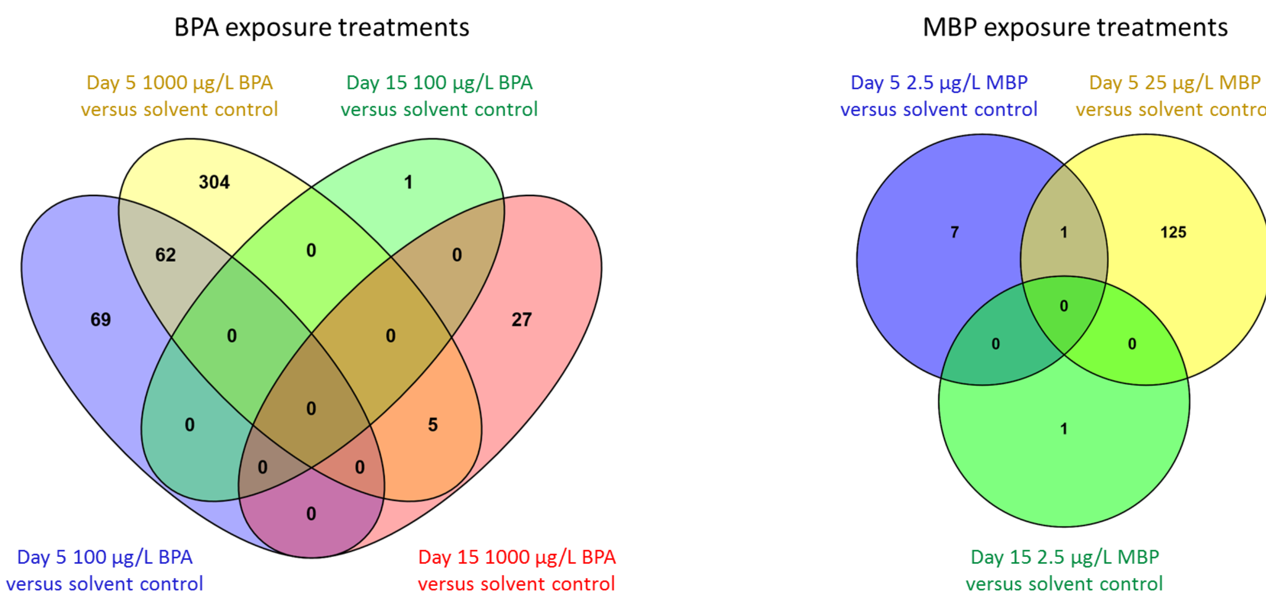


Figure S6: Venn diagrams showing overlap in differentially expressed genes in zebrafish (versus respective solvent controls) for BPA and for MBP exposure treatments

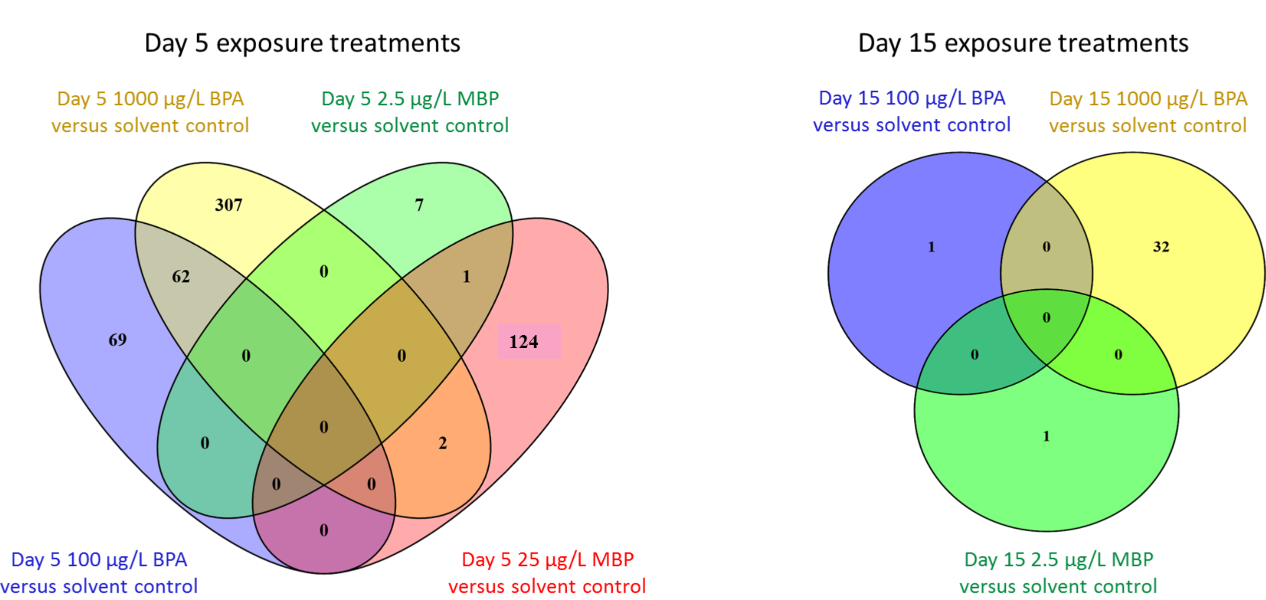


Figure S7: Venn diagrams showing overlap in differentially expressed genes in zebrafish (versus respective solvent controls) for both BPA and MBP at 5 and 15 days post fertilisation (dpf)

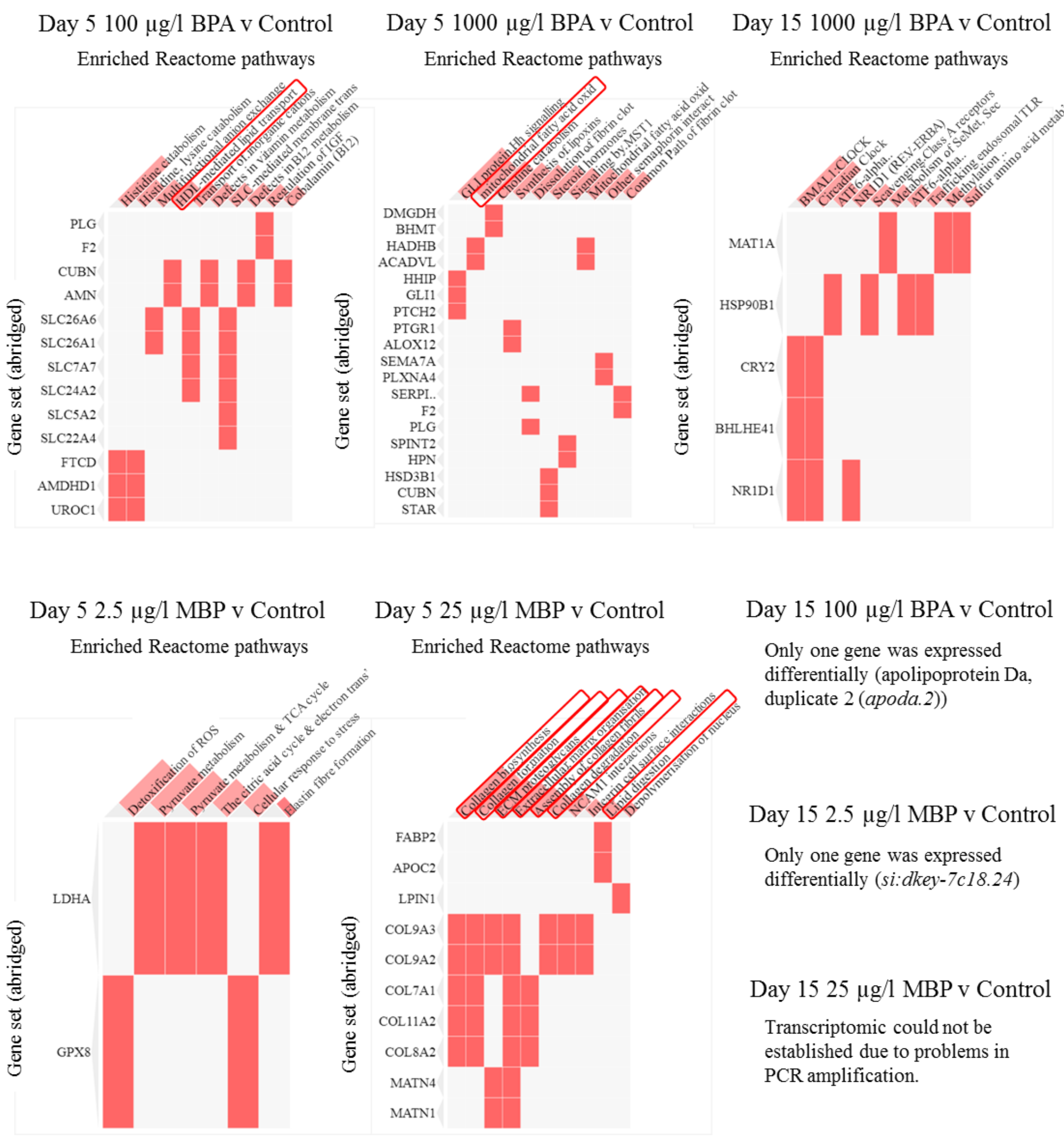


Figure S8: Gene set enrichment for Reactome pathways in heart tissues from 5 and 15 day old larval zebrafish in BPA and MBP exposure treatments (versus solvent controls).

Sequence data were generated from hearts pooled from ~30 individuals from each of 4 separate aquaria (nominally n=4 experimental replicates) per exposure treatment. Enriched pathways were identified using Enrichr and referenced to the Reactome database (2016). Pathways highlighted in red boxes are calcific aortic valve disease (CAVD) biomarkers.

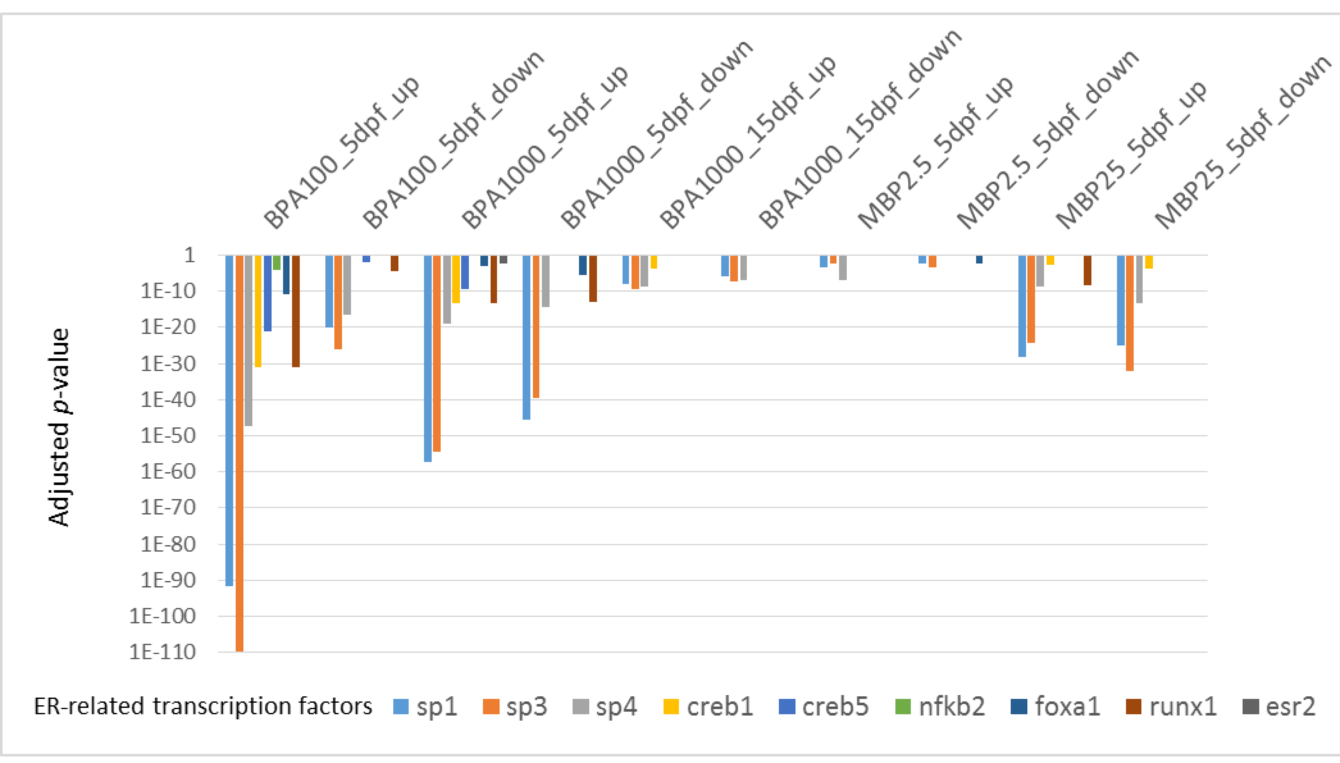


Figure S9: Enriched Transcription Factor Binding Site motifs 5 kB up- and down- stream of differentially expressed genes for BPA and MBP exposure treatments

Enriched transcription factor binding site motifs were identified using Analysis of Motif Enrichment (AME) in MEME suite 5.0.2. Enrichment is inversely proportional to adjusted *p*-value. Transcription factor binding site motifs associated with estrogen receptor signalling, included: estrogen receptor 2 (*esr2*); specificity proteins constituting ERE tethering factors (*sp1, sp3, sp4*); pioneer factors facilitating ERE binding (*foxa1, nfkb2, pbx1, runx1*); CAMP responsive element binding proteins (*creb1, creb5*).

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