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1 Supplementary Materials

1.1 Supplementary Figures and Tables

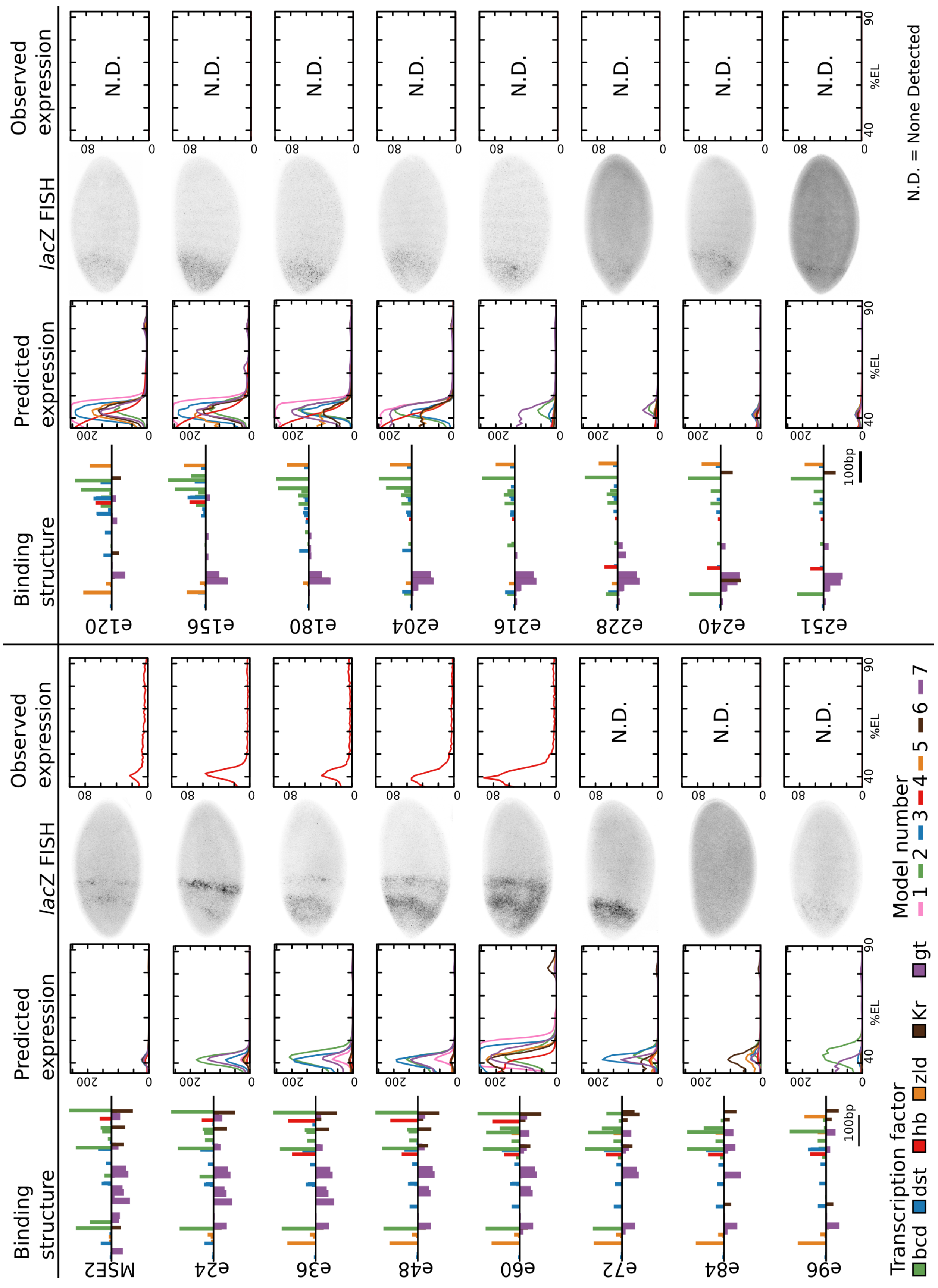

Figure S1. Continued on next page...

Figure S1. Expression and prediction on path to e251. Continued from previous page. For all tested enhancers the following are shown: (Column 1) Binding structure of tested enhancers. Activators are plotted on the positive y-axis and repressors on the negative. Bar height is proportional to the log-likelihood ratio of binding. (Column 2) The predicted output in each of 7 models used in the design of the sequences. (Column 3) The *lac*Z mRNA expression of a representative embryo. (Column 4) The quantitative level of mRNA expression for each line along a 10% DV stripe from 35.5% to 92.5% embryo length.

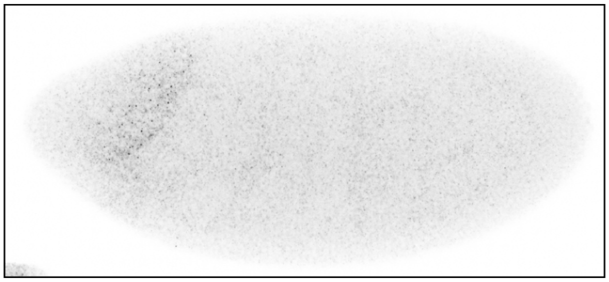

Figure S2. mRNA FISH of a reporter containing no enhancer. We generated a vector, as previously described, that contains no inserted putative enhancer and inserted the sequence into the Attp2 landing site. The resulting construct drives weak expression of an ectopic stripe anterior to *eve* stripe 1, confirming that this expression pattern is driven, at least in part, by sequences present on the vector.

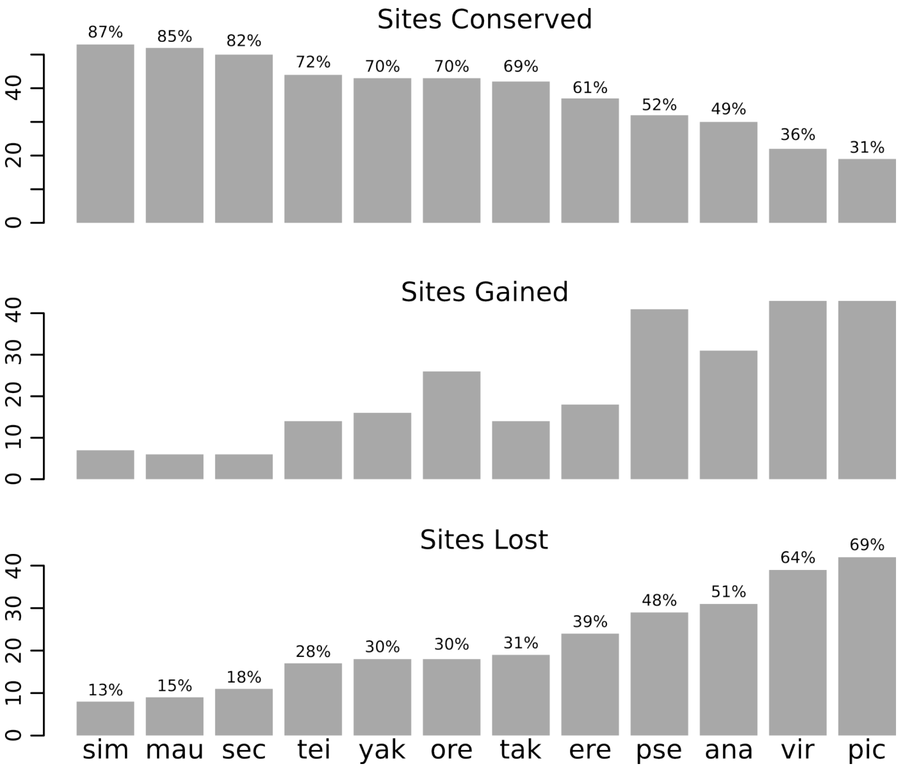

Figure S3. Conservation of motifs in S2Es. (Top) The number of binding motifs at LLR greater than 0 for the factors Bcd, hb, Kr, and gt that are conserved with S2E are shown for each putative S2E. The percent conservation is given above each bar. (Middle) The number of binding motifs (LLR>0) for the same 4 factors that are gained are shown for each putative S2E. (Bottom) The number of motifs (LLR>0) for the 4 factors that are lost are shown for each factor. The percent of sites lost is given above each bar. The sequences of each are reported in Kim et. al.[**?**].

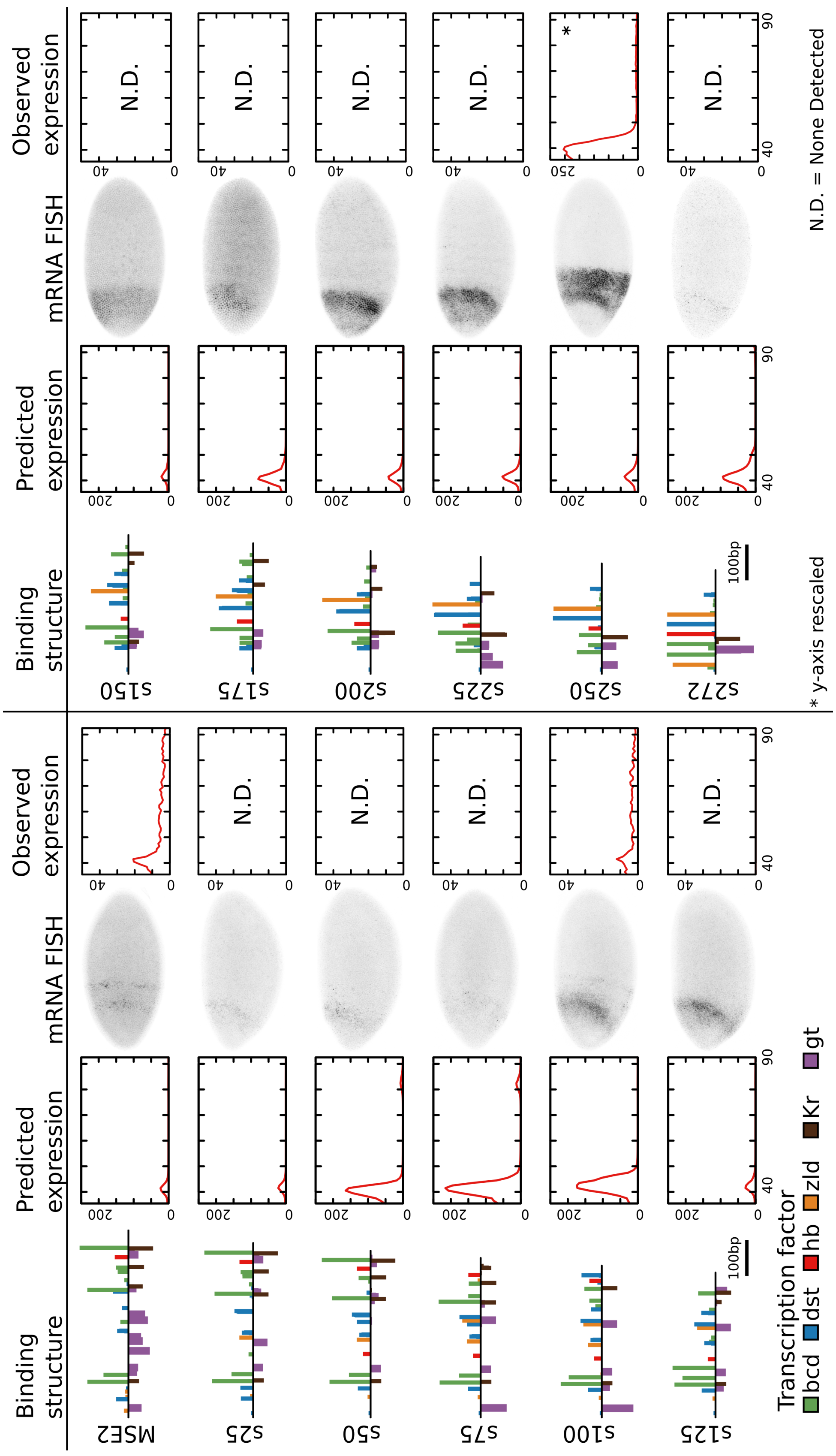

Figure S4. Continued on next page...

Figure S4. Expression and prediction along a path to s272. Continued from previous page. For all tested enhancers the following are shown: (Column 1) Binding structure of tested enhancers. Activators are plotted on the positive y-axis and repressors on the negative. Bar height is proportional to the log-likelihood ratio of binding. (Column 2) The predicted output in the model used in the design of the sequences. (Column 3) The *lac*Z mRNA expression of a represenative embryo. (Column 4) The quantitative level of mRNA expression for each line along a 10% DV stripe from 35.5% to 92.5% embryo length.

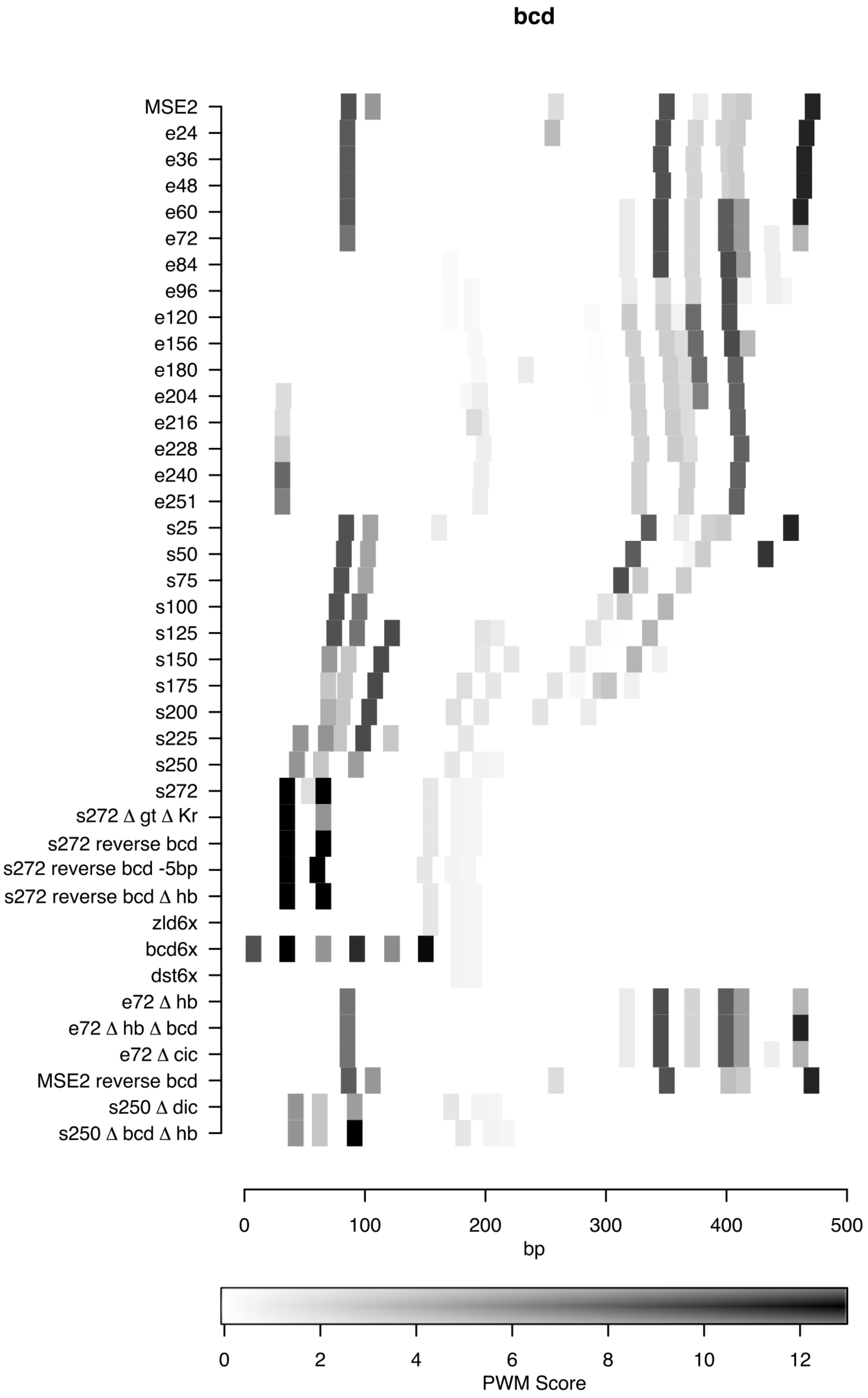

Figure S5. Binding sites for Bcd For each sequence used in this work, we calculated the Bcd PWM score at each position. Motif matches (score>0) are indicated by rectangles that are shaded according to the PWM score, with the legend under the x-axis.

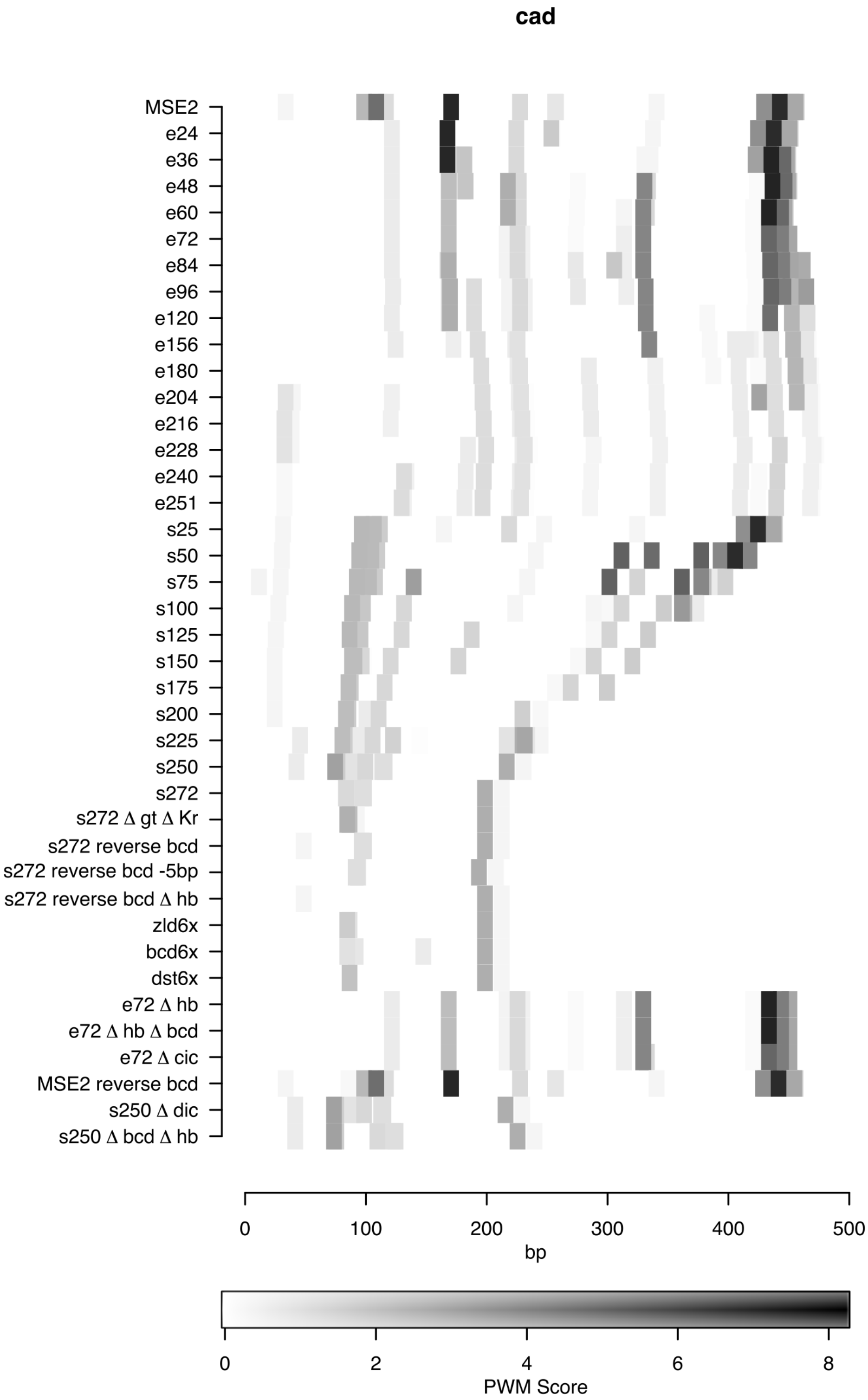

Figure S6. Binding sites for Cad For each sequence used in this work, we calculated the Cad PWM score at each position. Motif matches (score>0) are indicated by rectangles that are shaded according to the PWM score, with the legend under the x-axis.

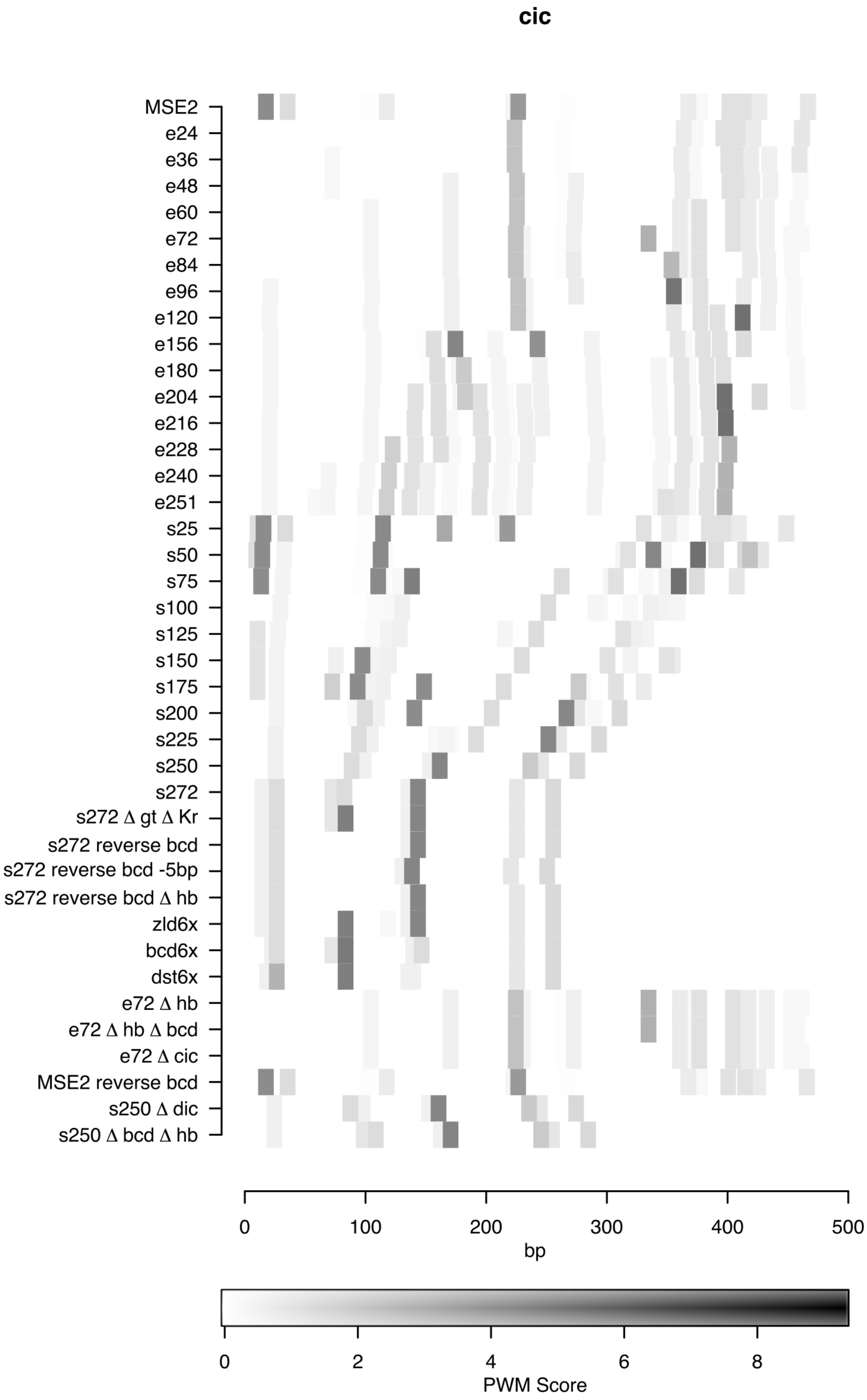

Figure S7. Binding sites for Cic For each sequence used in this work, we calculated the Cic PWM score at each position. Motif matches (score>0) are indicated by rectangles that are shaded according to the PWM score, with the legend under the x-axis.

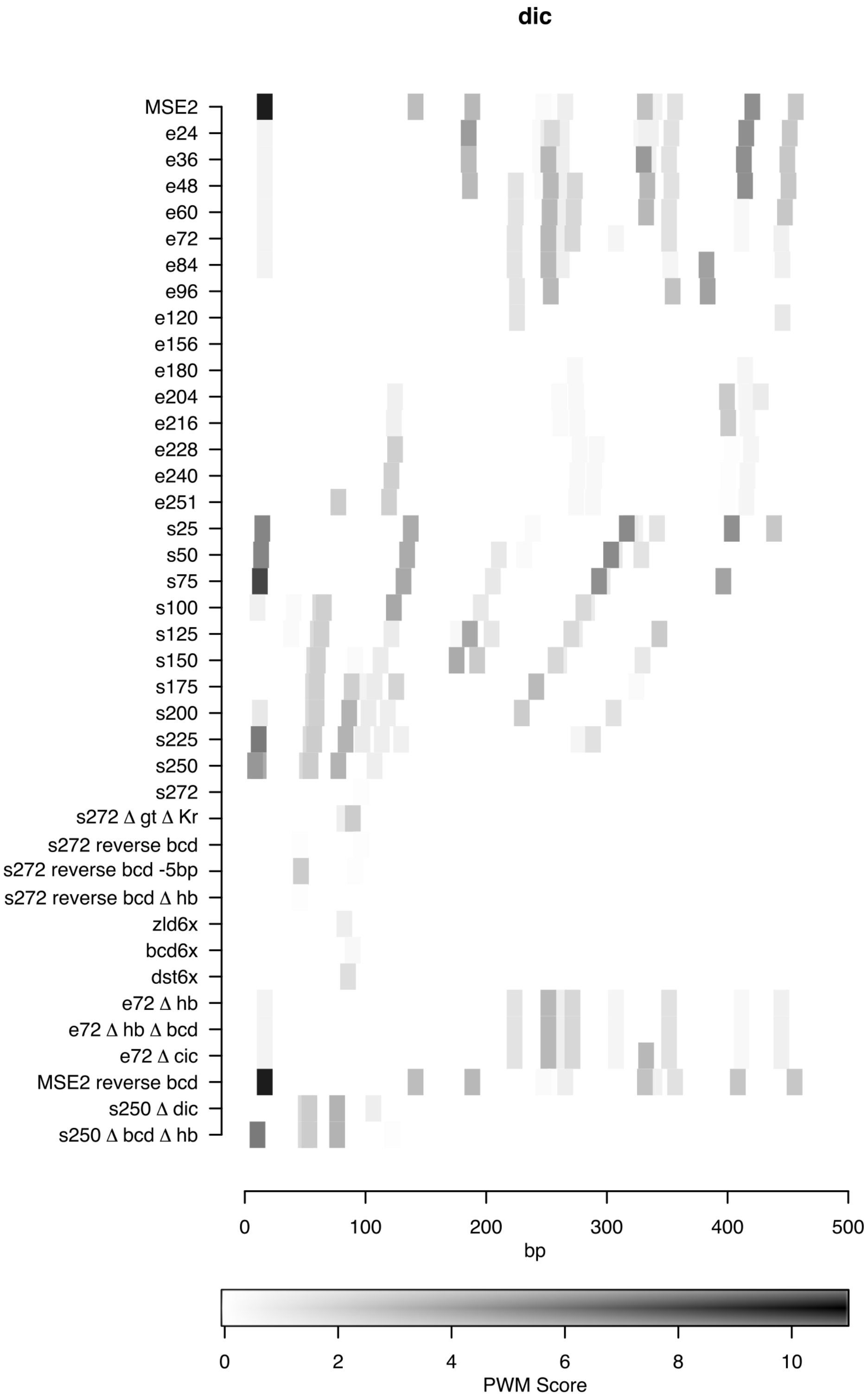

Figure S8. Binding sites for Dic For each sequence used in this work, we calculated the Dic PWM score at each position. Motif matches (score>0) are indicated by rectangles that are shaded according to the PWM score, with the legend under the x-axis.

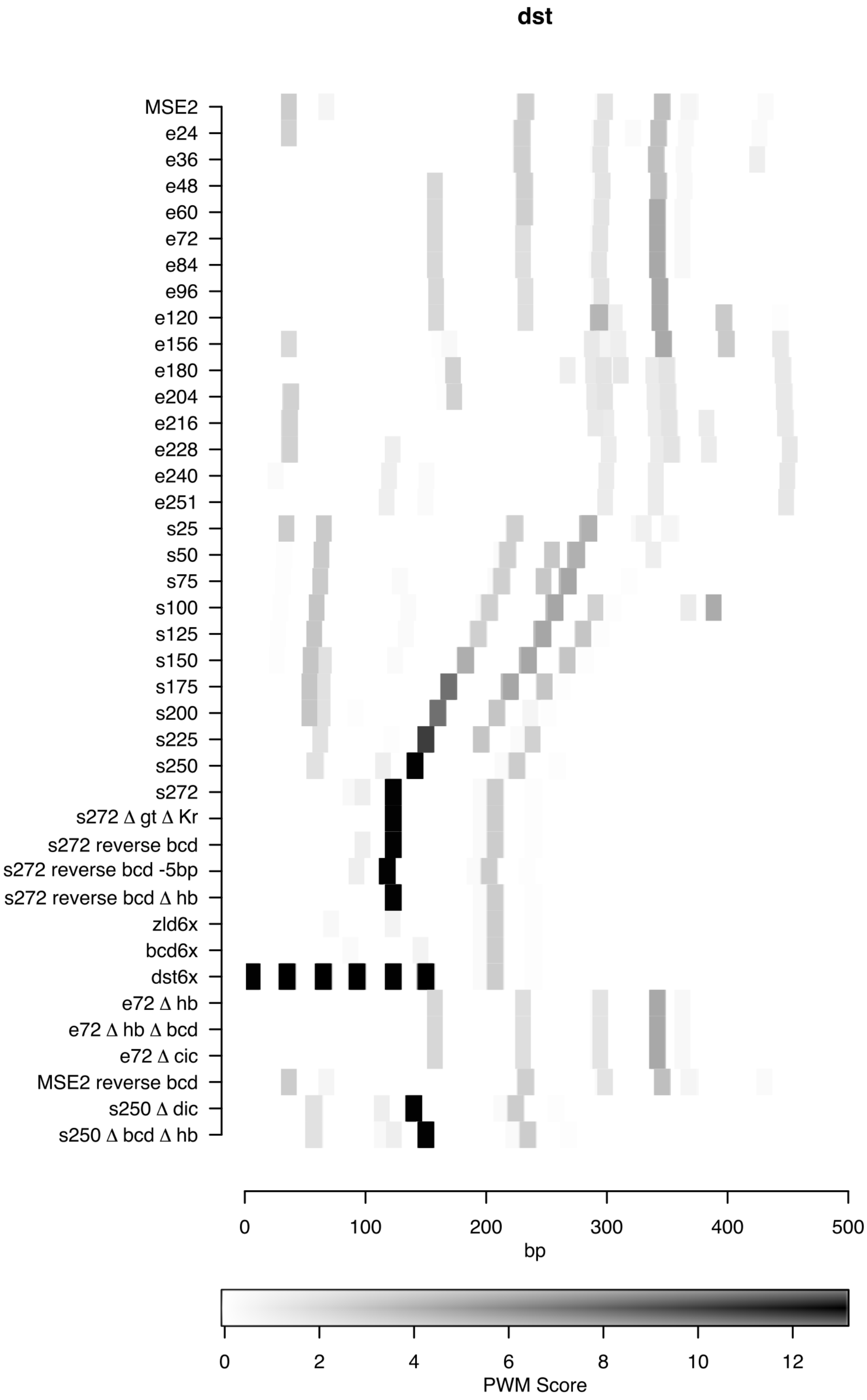

Figure S9. Binding sites for Dst For each sequence used in this work, we calculated the Dst PWM score at each position. Motif matches (score>0) are indicated by rectangles that are shaded according to the PWM score, with the legend under the x-axis.

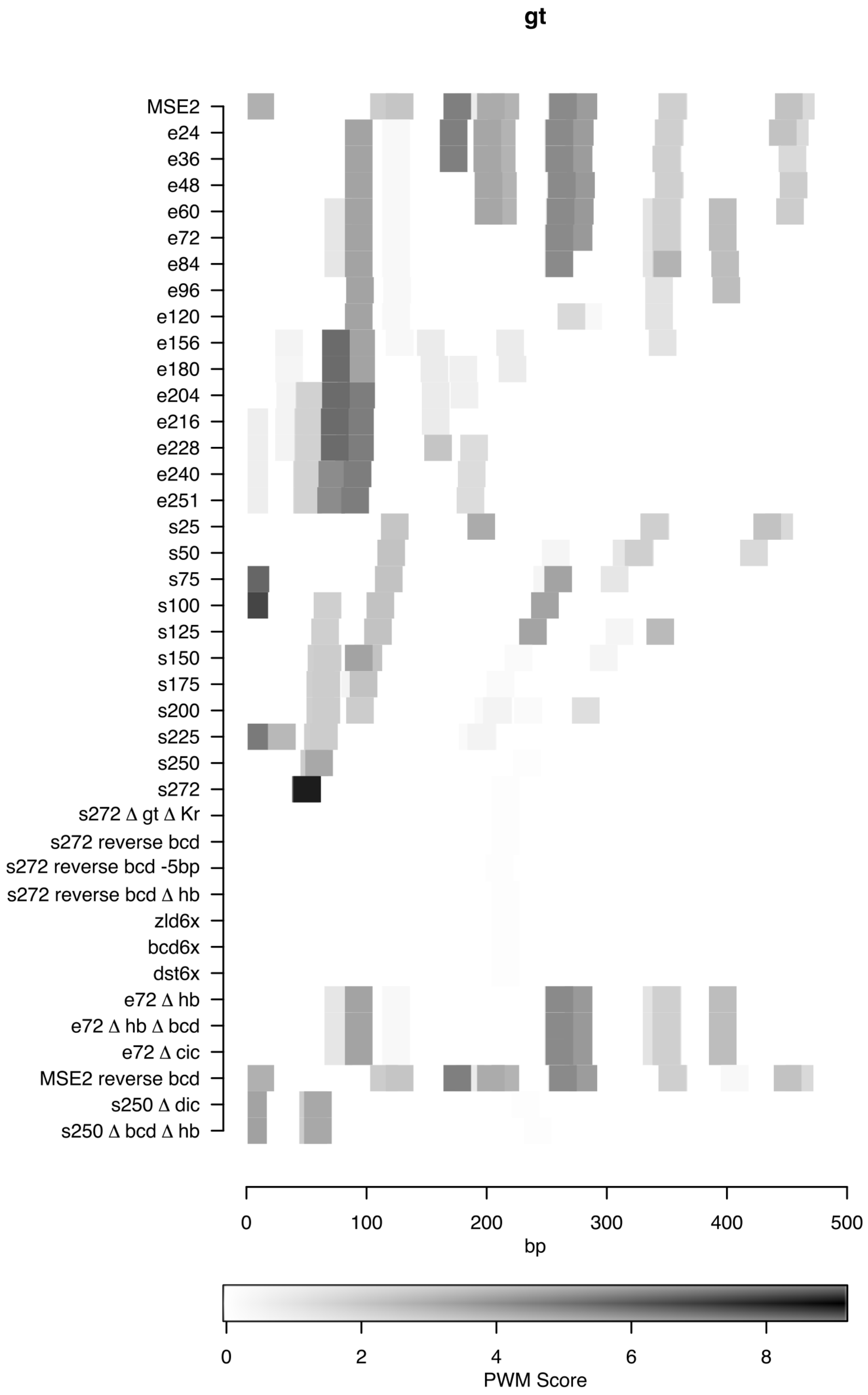

Figure S10. Binding sites for Gt For each sequence used in this work, we calculated the Gt PWM score at each position. Motif matches (score>0) are indicated by rectangles that are shaded according to the PWM score, with the legend under the x-axis.

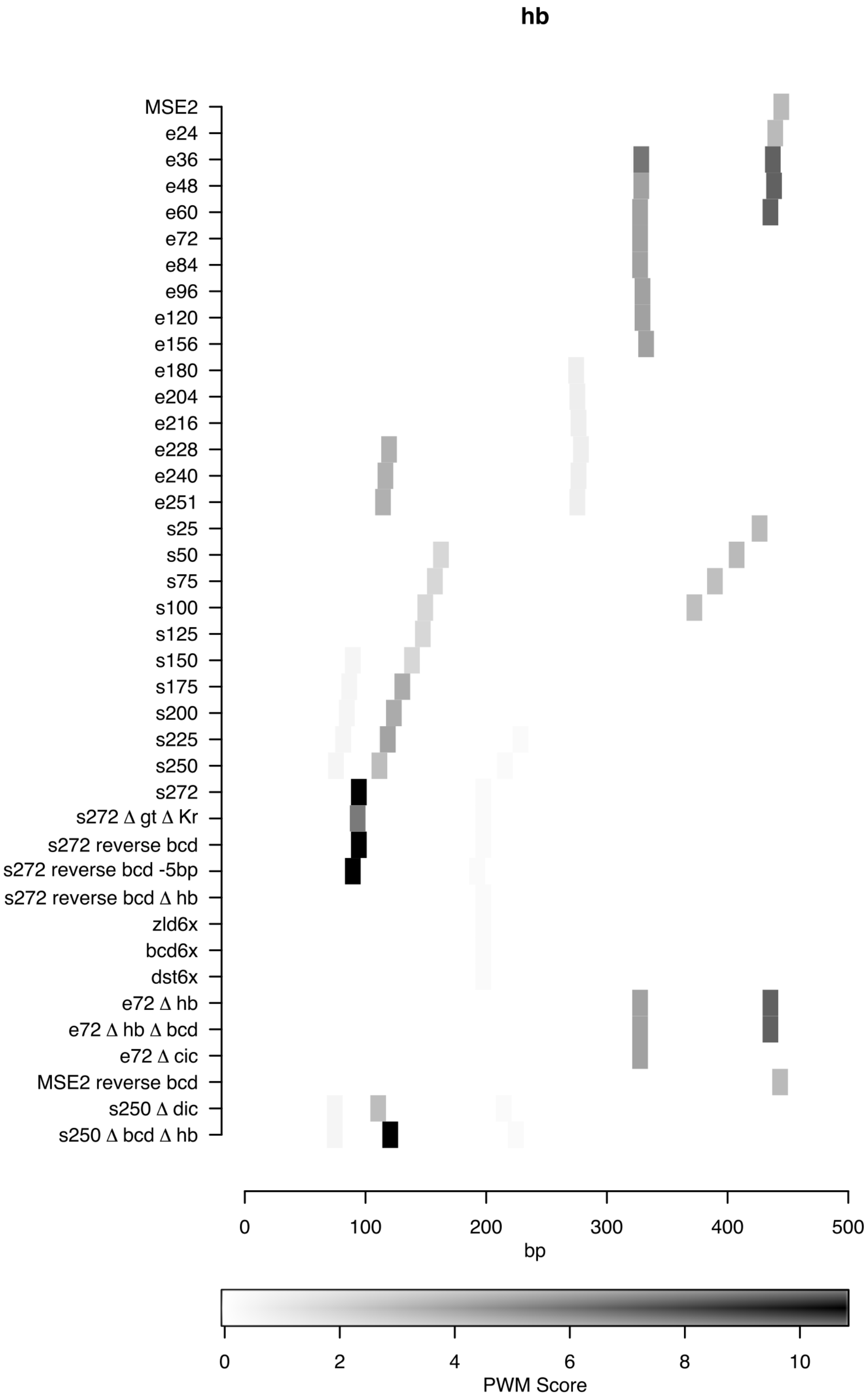

Figure S11. Binding sites for Hb For each sequence used in this work, we calculated the Hb PWM score at each position. Motif matches (score>0) are indicated by rectangles that are shaded according to the PWM score, with the legend under the x-axis.

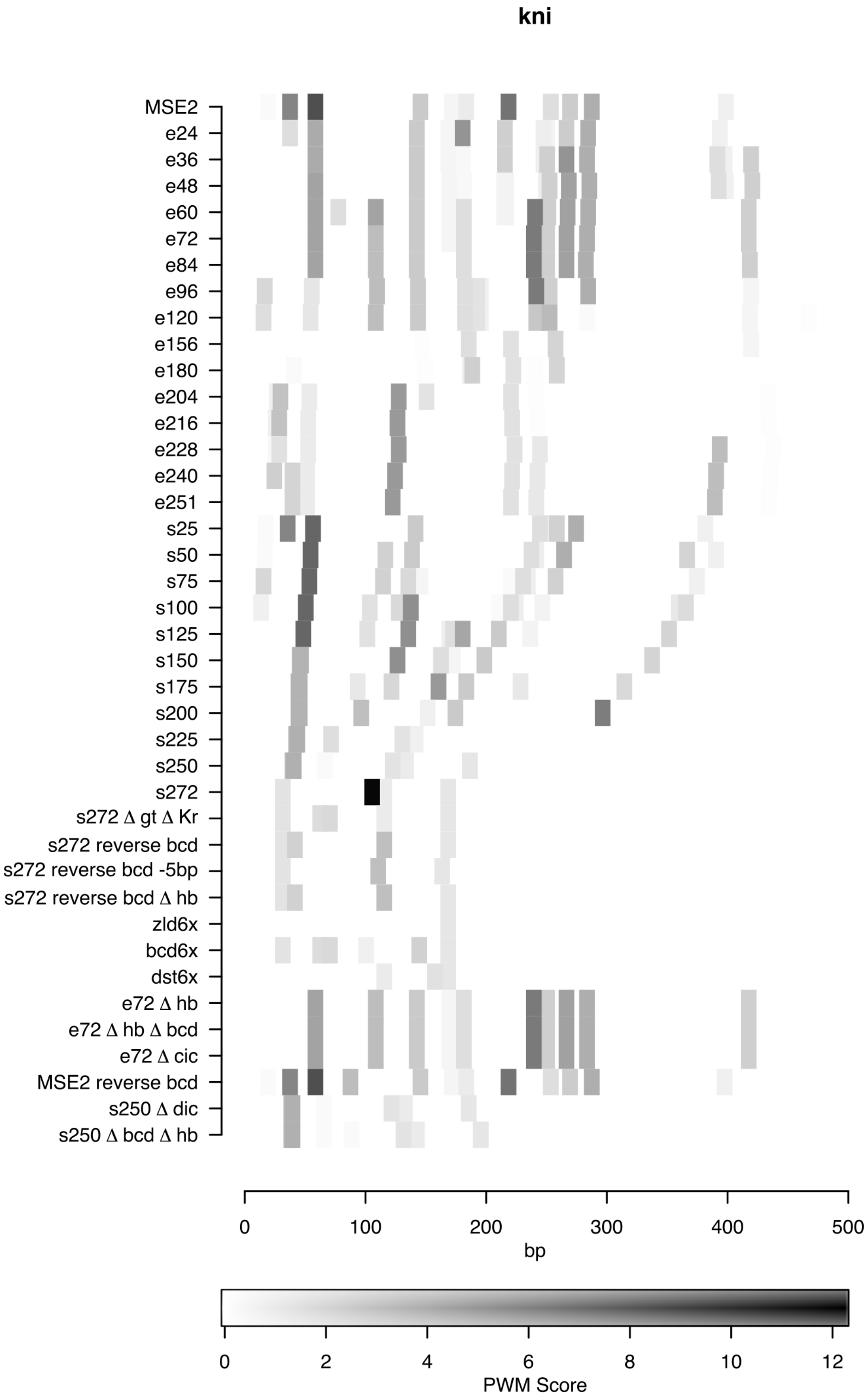

Figure S12. Binding sites for Kni For each sequence used in this work, we calculated the Kni PWM score at each position. Motif matches (score>0) are indicated by rectangles that are shaded according to the PWM score, with the legend under the *x*-axis.

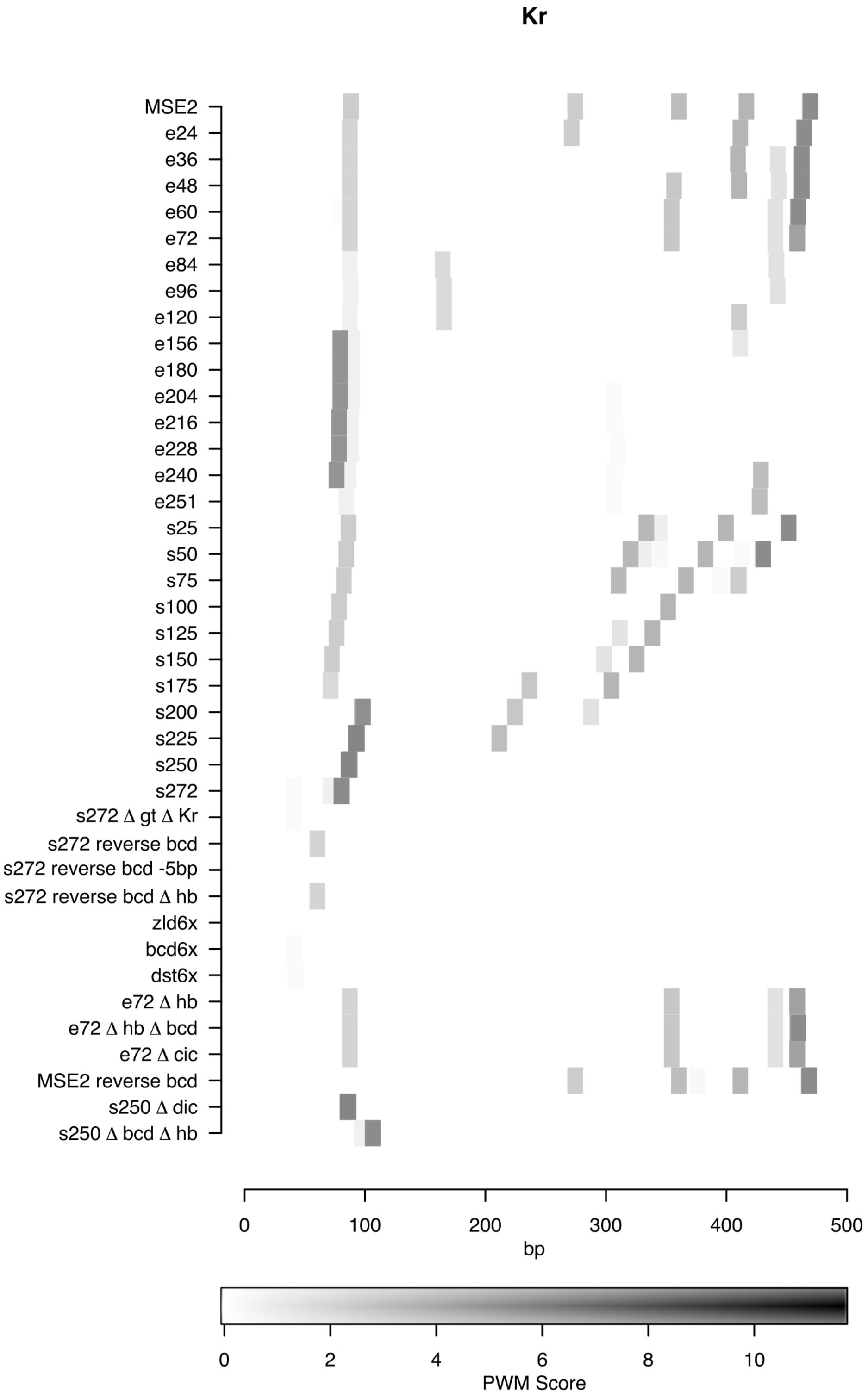

Figure S13. Binding sites for Kr For each sequence used in this work, we calculated the Kr PWM score at each position. Motif matches (score>0) are indicated by rectangles that are shaded according to the PWM score, with the legend under the x-axis.

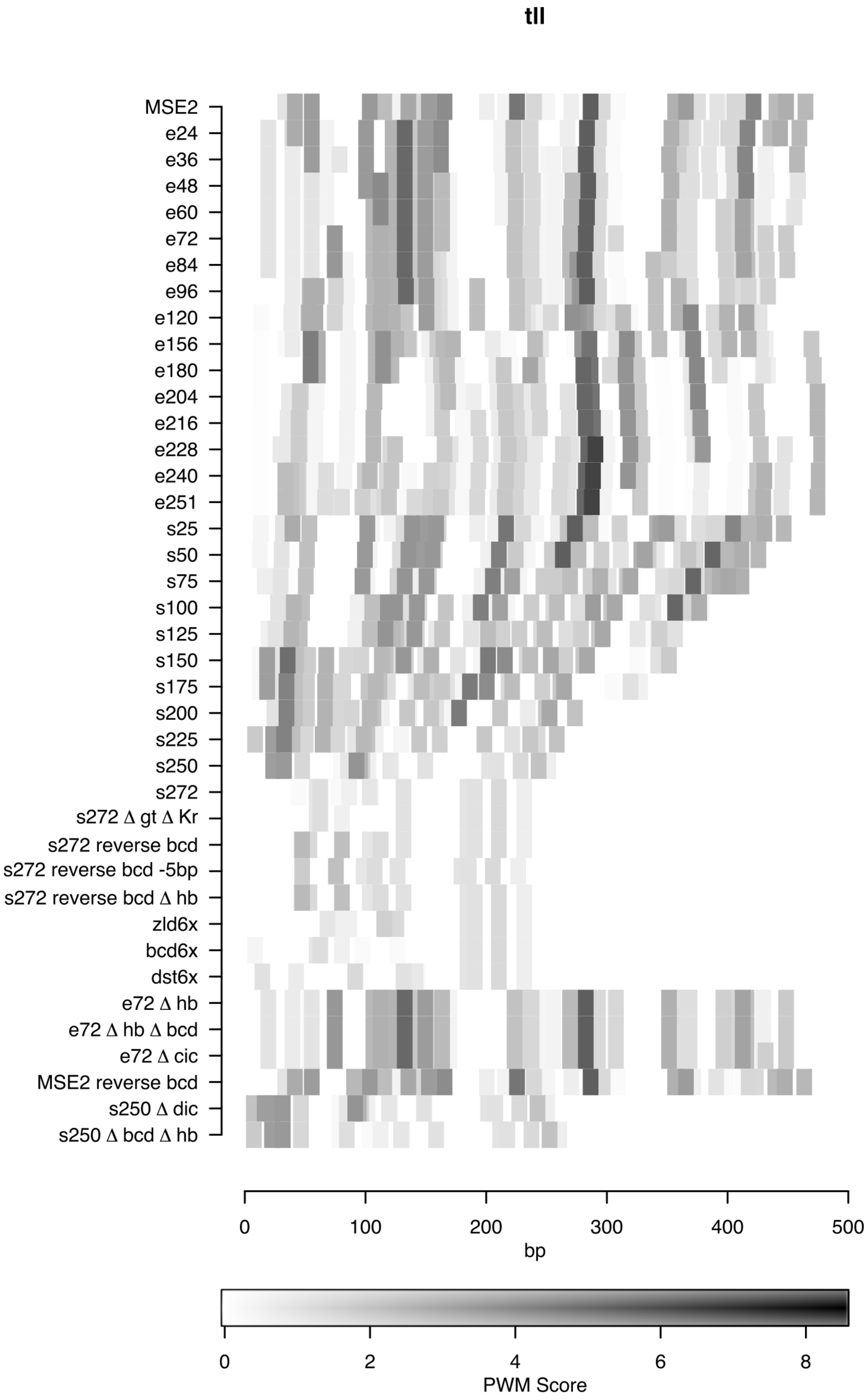

Figure S14. Binding sites for Tll For each sequence used in this work, we calculated the Tll PWM score at each position. Motif matches (score>0) are indicated by rectangles that are shaded according to the PWM score, with the legend under the x-axis.

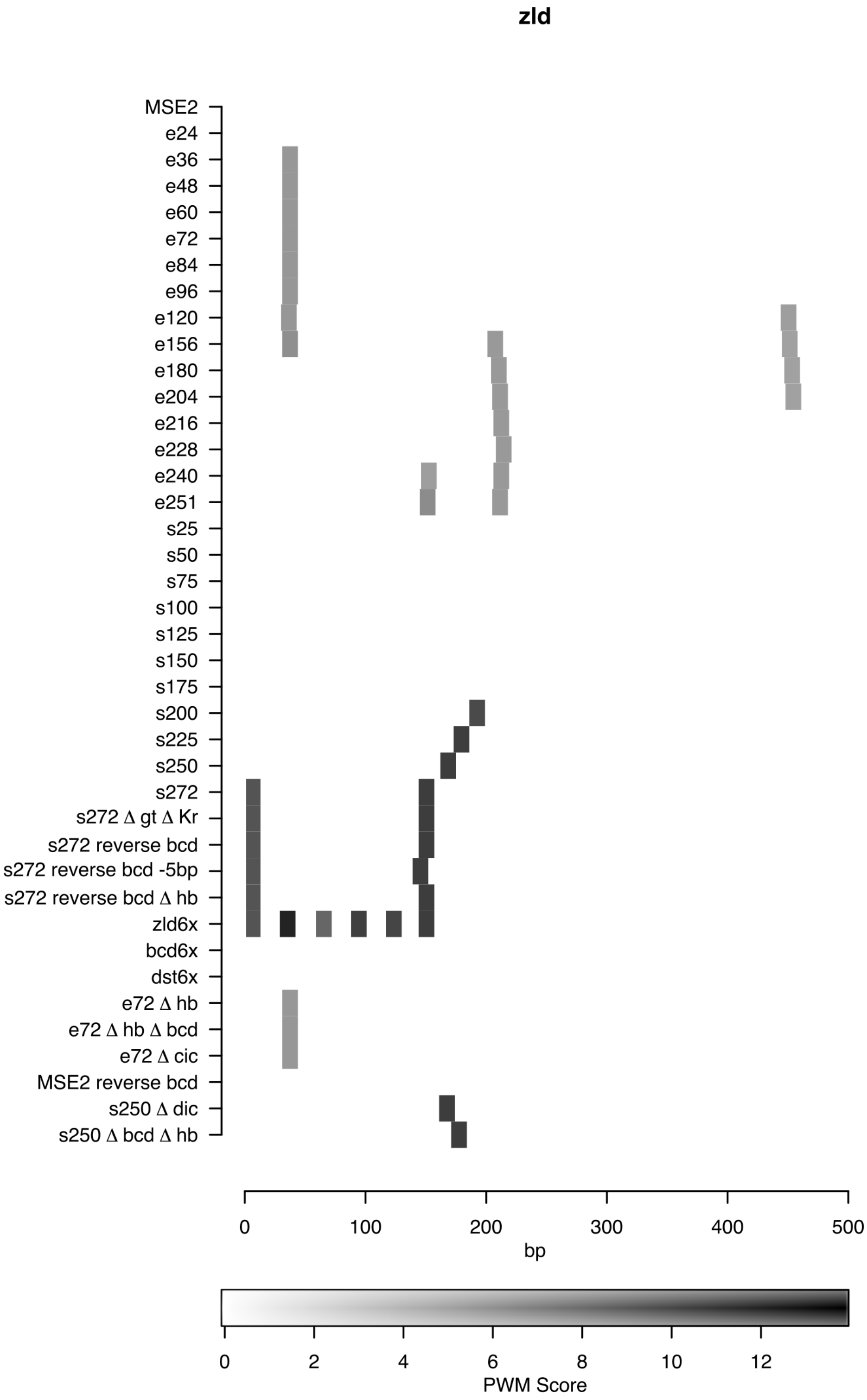

Figure S15. Binding sites for Zld For each sequence used in this work, we calculated the Zld PWM score at each position. Motif matches (score>0) are indicated by rectangles that are shaded according to the PWM score, with the legend under the x-axis.

		Model						
Parameter	TF	1	2	3	4	5	6	7
A	Bicoid	0.024022	0.037788	0.076132	0.002134	0.089843	0.063042	0.489863
λ	Bicoid	1.934457	4.992031	2.162269	0.591795	0.590000	0.590000	3.441042
E^A	Bicoid	2.677410	0.062923	0.000149	16.694600	0.000135	0.000118	0.000108
E^C	Bicoid	0.999878	0.217722	0.350294	0.683285	0.235757	0.194850	0.139848
D^C	Bicoid	150.660000	158.192000	150.630000	182.596000	186.889000	183.882000	184.813000
ω	Bicoid	499.457000	189.210000	127.107000	34.655300	43.989600	499.673000	9.992570
Threshold	Bicoid	1.710000	1.710000	1.710000	-1.200000	-1.000000	-1.000000	1.710000
PWM	Bicoid	Bcd_selex	Bcd_selex	Bcd_selex	bicoid_mitomi	bicoid_mitomi	bicoid_mitomi	Bcd_selex
A	Caudal	0.071453	0.052367	0.026425	0.018378	0.003403	0.008447	0.014319
λ	Caudal	4.998159	4.976927	3.185278	9.990996	4.999857	4.999639	4.996867
E^A	Caudal	0.000103	0.000115	0.000107	0.000136	0.000111	0.000104	0.000153
E^C	Caudal	0.999899	0.337226	0.887749	0.999925	0.999946	0.999940	0.512709
D^C	Caudal	16.931600	70.875200	22.309700	11.549400	69.292800	36.338200	69.615400
Threshold	Caudal	2.501270	3.061023	2.065076	2.992003	2.108353	2.163674	2.728832
A	Stat92E	3.990583	1.099451	3.999120	3.999022	3.999657	3.998975	3.999516
λ	Stat92E	0.894867	2.585787	0.696684	0.693574	0.743904	0.735156	1.309302
E^A	Stat92E	0.000193	0.000154	19.995300	19.996900	19.999800	19.999000	0.000392
Threshold	Stat92E	4.038679	2.836359	3.631432	2.977216	3.589654	4.098801	3.890423
A	Dicheate	3.953880	3.893817	0.179983	0.051160	3.991435	0.271187	1.163608
λ	Dicheate	1.310409	1.983447	4.548029	2.484454	0.929774	4.992717	2.516001
E^A	Dicheate	0.000175	0.000139	0.453824	0.000812	0.282053	0.000156	0.000108
Threshold	Dicheate	4.003251	4.794568	2.962805	3.813067	4.909050	4.384482	2.416283
A	Hunchback	0.089845	2.724241	0.041525	0.046542	0.059710	0.054422	1.172800
λ	Hunchback	4.735283	1.836818	4.999943	0.591793	4.554233	4.999490	1.839149
E^Q	Hunchback	0.969302	0.999983	0.999964	0.999967	0.999998	0.999987	0.999985
E^A	Hunchback	19.546500	20.417400	29.999000	18.683900	24.630300	27.349300	29.979000
E^D	Hunchback	0.441831	0.323916	0.581803	0.976922	0.095546	0.000209	0.490470
Threshold	Hunchback	0.630000	0.630000	0.630000	-3.250000	0.630000	0.630000	0.630000
PWM	Hunchback	Hb_selex	Hb_selex	Hb_selex	Hb_mitomi	Hb_selex	Hb_selex	Hb_selex
A	Zelda	NA	NA	NA	0.066168	NA	NA	0.002172
λ	Zelda	NA	NA	NA	9.999855	NA	NA	3.755900
E^A	Zelda	NA	NA	NA	0.558206	NA	NA	19.982600
Threshold	Zelda	NA	NA	NA	1.487750	NA	NA	-0.754056
A	Kruppel	3.997768	0.038668	2.952886	0.865115	0.025163	0.058992	0.055444
λ	Kruppel	0.888617	4.040318	0.980777	1.266060	4.997118	2.863341	4.999282
E^Q	Kruppel	0.999988	0.902770	0.999996	0.999938	0.999990	0.999966	0.999997
E^D	Kruppel	0.999654	0.999748	0.999654	0.261343	0.414812	0.724846	0.758756
Threshold	Kruppel	0.335286	2.116363	0.072662	1.348795	0.445604	0.536644	0.409664
A	Knirps	0.208940	2.233074	0.272310	0.060819	0.267202	0.396735	0.094753
λ	Knirps	4.998806	2.487536	1.568829	3.879400	1.908455	1.441430	3.153326
E^Q	Knirps	0.168820	0.063714	0.999168	0.999906	0.999376	0.999826	0.999686
E^D	Knirps	0.000515	0.123624	0.999541	0.089845	0.378947	0.034038	0.958817
Threshold	Knirps	4.321854	2.234361	4.856997	5.611598	4.105547	3.739100	2.262279
A	Giant	0.125367	2.530848	0.039886	0.110539	3.996551	0.054890	3.995412
λ	Giant	4.998344	1.713581	4.999174	2.321564	1.273039	4.999384	1.382071
E^Q	Giant	0.386106	0.723628	0.739607	0.795631	0.492502	0.426621	0.858064
E^D	Giant	0.974631	0.171351	0.999683	0.000246	0.999547	0.999945	0.171011
Threshold	Giant	0.585501	0.591202	0.504367	2.611903	0.723453	0.646484	0.653197
A	Tailless	0.422458	0.023573	1.946981	0.013529	1.014708	0.282655	0.063704
λ	Tailless	1.304139	4.980396	0.962922	9.761793	1.132544	1.491165	2.710489
E^Q	Tailless	0.999524	0.999485	0.999960	0.000136	0.999794	0.998830	0.999869
E^D	Tailless	0.000607	0.000255	0.000155	0.000168	0.000241	0.000186	0.000167
Threshold	Tailless	1.968527	1.977264	1.972201	2.506890	1.812958	1.969305	1.974659
θ	NA	6.147710	6.085620	5.955810	7.478010	5.727320	5.731930	6.242990

Table S1. Continued on next page...

Table S1. Continued from previous page. The model parameters and PWMs that were used generate the sequences in this work are given. All parameters are as described in Kim et. al.[?] and models 1,2, and 3 are discussed in that work where they are called Model 01, Model 06, and Model 07 repectively. Models 5, 6, and 7 were trained as descibed in Kim et. al [?]. Model 4 was trained as described in Martinez et. al [?]. PWMs are specified for factors where the PWM chosen was different among fits.

1.2 Position Weight Matrices Used

>	Bcd_selex													
A	83	74	108	48	5	381	379	4	0	5	72	61	65	68
C	114	159	127	149	0	0	0	0	383	340	136	174	166	158
G	106	72	114	11	0	1	4	4	0	2	132	60	52	49
T	80	78	34	175	376	0	0	374	0	34	43	88	100	108

>	cad_1hyb									
A	9	12	2	3	12	38	0	3	21	1
C	9	6	3	0	0	0	0	0	0	8
G	4	3	3	0	1	0	0	7	14	10
T	11	16	29	34	24	0	38	27	0	1

>	dst_selex											
A	0	1	2	1	0	5	3	0	24	28	27	5
C	0	0	1	27	20	16	3	1	3	0	1	7
G	0	1	0	1	6	8	22	27	1	1	0	6
T	28	28	27	1	3	1	2	0	2	1	2	11

>	dic_1hyb										
A	1	0	0	20	0	0	2	0	1	3	6
C	7	25	17	0	0	0	0	0	2	10	1
G	7	0	0	0	0	3	27	0	4	6	1
T	13	3	1	9	28	26	0	28	22	9	21

>	hb_selex									
A	53	1	0	1	0	0	0	280	31	20
C	6	5	1	0	1	2	1	0	43	100
G	224	3	0	0	0	0	0	2	78	109
T	7	279	288	288	288	287	288	5	138	61

>	Kr_selex									
A	17	187	158	0	1	0	8	0	2	44
C	73	5	39	194	194	197	22	1	34	109
G	6	0	0	0	0	0	6	0	1	15
T	101	5	0	2	2	0	161	195	159	29

>	kni_1hyb											
A	19	25	16	5	0	21	0	17	1	0	25	5
C	0	0	0	9	4	0	0	0	3	26	0	12
G	1	0	0	6	0	5	26	7	18	0	0	7
T	3	0	10	5	21	0	0	1	4	0	0	2

>	gt_selex								
A	85	11	776	8	82	0	1020	1105	15
C	61	107	24	762	19	555	88	0	378
G	18	359	275	64	996	0	0	0	85
T	941	630	32	274	11	553	0	2	631

> tll_matrix

A	11	1	1	5	1	11	1	0	0
C	7	2	2	1	2	1	17	2	2
G	0	2	1	0	15	5	0	1	2
T	0	15	16	14	0	3	2	17	15

> zld_1hyb

A	54	7	756	0	1	4	756	76
C	0	749	1	0	0	0	5	75
G	478	0	1	731	750	3	0	566
T	229	5	3	30	10	754	0	44

> Bcd_mitomi

A	0.4446859	1.024804	0.000000	1.308060	1.212380	0.0000000
C	0.8241404	1.212753	0.901402	1.386241	0.819161	1.1815924
G	0.0000000	0.000000	1.211622	1.455025	1.407177	1.1908577
T	0.8472205	1.141426	1.261173	0.000000	0.000000	0.8257633

> Hb_mitomi

A	0.2557999	1.8157733	2.896855	2.954505	3.928484	3.637427	1.759458	0.0000000
C	0.6691087	0.8469678	2.268623	1.556663	4.230409	2.748917	3.135718	1.5068606
G	0.0000000	2.5883169	2.128065	1.824146	3.205319	3.789288	2.927774	0.8407924
T	0.2229569	0.0000000	0.000000	0.000000	0.000000	0.000000	0.000000	1.4966308

> cic_1hyb

A	1	0	0	18	0	0	0	18
C	10	11	18	0	0	0	10	0
G	0	0	0	0	0	0	8	0
T	6	7	0	0	18	18	0	0

1.3 Sequences

The sequences generated in this work are given below.

>MSE2

CCGGTACTGCATAACAATGGAACCCGAACCGTAACTGGGACAGATCGAAAAGCTGGCCTGGTTTCTCGCTGTGTGTGCCGTGTT
AATCCGTTTGCCATCAGCGAGATTATTAGTCAATTGCAGTTGCAGCGTTTCGCTTTCGTCCTCGTTTCACTTTCGAGTTAGACTTTAT
TGCAGCATCTTGAACAATCGTCGCAGTTTGGTAACACGCTGTGCCATACTTTCATTTAGACGGAATCGAGGGACCCTGGACTATAAT
CGCACAACGAGACCGGGTTGCGAAGTCAGGGCATTCCGCCGATCTAGCCATCGCCATCTTCTGCGGGCGTTTGTTTGTTTGTTTGCT
GGGATTAGCCAAGGGCTTGACTTGGAATCCAATCCCGATCCCTAGCCCGATCCCAATCCCAATCCCAATCCCTTGTCCTTTTCATTAG
AAAGTCATAAAAACACATAATAATGATGTCGAAGGGATTAGGGG

>e24

CCGGTACTGAATACCAGTGGATTCCGAACCGTAACAGGGACAGATCGAAAAGCTGGCCTGGTATCTGCTGTGTGTGCCGTGTT
AATCCGTTGCCACAGCGAGATTGTTAGTCACTTGCAGTTACAGTATTTCGCTGTCGTCCTCGTTTCACTTTCGAGTTAGACTTTATT
GCAGCATCTAGAACAATCGTCGCAGTTCGGTAACACGCTGTGCCATACTATCATTTAGACGGAATCGAGGGACCCTGGACCATAATC
GCACAACGAGACCGGGTTGCGAAGTCAGGGCATTCCGCCGATCTAGCCATCGCCATCTTCTCCGGGTGTTTGTTTGTTTGTTTGCTG
GGATTAGCCAAGGGCTGACTTGGAATCCAATCCCGTCCCTAGCCCGATCCCAATCCCAATCCCAATCCCTTGTCCTTTTCATTAGAAA
GTCATAAAAACACATAATAATGATGTCGAAGGGATTAGGGG

>e36

CCGGTACTGAATACCAGTGGATTCCGAACCGTAACAGGTACAGATCGAAAAGCTGGCCTGGTATCTGCTGTGTGAGCCGTGTTA
ATCCGTTGCCACAGCGAGATTGTTAGTCACTTGCAGTTACAGTATTTCGCTGTCGTCCTCGTTTCACTTTCGAGTTAGACTTTATTGC
AGCATCTATAACAATCGTCGCAGTTCGGTAACACGCTGTGCCATACTATCATTTAGACGGAATCGAGGGACCCTGGACCATAGTCGC
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TAAAAACCCATAATAATGATGTCGAAGGGATTAGGGG

>e48

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GCAGCATCTATAACAATCGTCGCAGTTCGGTAACACGCTGTGCCATATCTATCATTTAGACGGAATCGAGGGACCCTGGACCATAGT

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GATTAGCCAAGGGTGACTTGGAATCCAATCCCGTCCCTAGCCCGATCCCGATCCCAATCCCAATCCCTTGTCCTCTTCATTTGAAACT
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>e60
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GCACAACGAGACCAATTGCGAAGTCAGGGCATTCCGCCGATCTAGCCATCGCCATCTTATCCGCGTGTTTTATGATTGTTTGCGGGA
TTAGCCAAGGGTGACTTGGAATCCAATCCCGTCCATAGCCCGATCCCGACCTAATCCCAATCCCTAGTCCTCTTCATTTGAAACTCAT
AAAAACCCATAATAATGATGCGAAGGGATTAGGGG
>e72
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