Supplemental Information | Snetkova et al. *Ultraconserved Enhancer Function Does Not Require Perfect Sequence Conservation.*

Supplemental Tables

Table S1: Loss of function results from ultraconserved enhancer mutagenesis. P-values and FASTA sequences of reference and mutated alleles (provided separately).

Table S2: Gain of function results from ultraconserved enhancer mutagenesis. P-values and FASTA sequences of reference and mutated alleles (provided separately).

Table S3. Details for hs122 mutated enhancer knock-in animals phenotyped for dentate gyrus abnormalities.

Mouse line	**Sample (mouse tag#)**	**Genotype**	**Age**	**Sex**	**Dentate Gyrus Length (µm)**
hs122mut	E1594	Wild-type	P32	Male	1962.08699
	E1596	Wild-type	P32	Male	2126.84771
	E1756	Wild-type	P41	Male	1950.04569
	E1595	Knock-in	P32	Male	1642.86569
	E1598	Knock-in	P32	Male	1611.85219
	E1755	Knock-in	P41	Male	1598.53229
	E1757	Knock-in	P41	Male	1569.72977

Ages are given in days after birth. Measurements of dentate gyrus length for each sample are the means from multiple measured sections (see **Methods**). For the plot in **Figure 5b**, measurements for knock-in animals were normalized by wild-type littermates. Raw measurements are plotted in **Extended Data Fig. 9**.

Table S4. Details for hs121 mutated enhancer knock-in animals phenotyped for abnormalities in VIP+ interneuron populations.

Mouse line	Sample (mouse tag#)	Genotype	Enhancer Activity of the Mutant Allele in the Transgenic Assay	Age	Sex	Cell density (cells/mm^2)
hs121mut1	E1675	Wild-type	N/A	P46	Male	60.87
	E1679	Wild-type	N/A	P46	Male	56.83
	E1674	Knock-in	Active	P46	Male	58.41
	E1676	Knock-in	Active	P46	Male	58.96
	E1749	Wild-type	N/A	P49	Male	64.24
	E1746	Knock-in	Active	P49	Male	67.76
	E1747	Knock-in	Active	P49	Male	59.81
hs121mut2	E1545	Wild-type	N/A	P67	Male	54.88
	E1546	Wild-type	N/A	P67	Male	52.53
	E1544	Knock-in	Inactive	P67	Male	67.47
	E1547	Knock-in	Inactive	P67	Male	65.25
	E1737	Wild-type	N/A	P56	Male	68.50
	E1739	Knock-in	Inactive	P56	Male	70.25
	E1740	Knock-in	Inactive	P56	Male	81.07

Ages are given in days after birth. Measurements of cell densities for each sample are the means from multiple measured sections (see **Methods**). For a plot in **Figures 6b** measurements for knock-in animals were normalized by wild-type littermates. Raw measurements are plotted in **Extended Data Fig. 10**.

Table S5: Primers used to amplify enhancers to make transgenic enhancer-reporter mice (provided separately).

Table S6: Primers used to generate and characterize hs122 and hs121 enhancer knock-in mice (provided separately).

Table S7. Knock-in founder mice generated for this study.

Enhancer	**Founders**		**Description**
hs122	E1239	Female, Heterozygous/ Mosaic for knock-in allele	Knock-in of enhancer allele with 5% of base pairs mutated in place of the reference hs122 allele. This mutant enhancer allele showed no activity in the transgenic assays. Allele generated by homology driven repair using a plasmid template with homologous arms.
hs122	E1245	Male, Hemizygous for knock-in allele	
hs122	E1254	Male, Hemizygous/ Mosaic for knock-in allele	
hs122	E1257	Female, Homozygous for knock-in allele	
hs122	E1263	Male, Hemizygous for knock-in allele	
hs121	D5859	Male, Hemizygous/Mosaic for knock-in allele	Knock-in of enhancer allele with 5% of base pairs mutated in place of the reference hs121 allele. Activity of this mutant enhancer allele in the transgenic assay was similar to the reference hs121 allele. Allele generated by homology driven repair using a plasmid template with homologous arms.
hs121	E1092	Female, Heterozygous/ Mosaic for knock-in allele	Knock-in of enhancer allele with 5% of base pairs mutated in place of the reference hs121 allele. This mutant enhancer allele showed no activity in the transgenic assays. Allele generated by homology driven repair using a plasmid template with homologous arms.

All knock-ins were generated in the FVB mouse strain. All founders were screened by Sanger sequencing of overlapping PCR fragments starting outside of the homologous recombination template. All founders were also tested for the homologous recombination template plasmid backbone, and only founders with a single integration of homologous recombination template were used for line propagation.

The complete Sanger sequence of an F1 male mouse hemizygous for the hs122 enhancer allele with 5% of base pairs mutated:

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TGGATTTTTTAAAAAATGTATACATTTCCCAATGTCTACATAAGTGATGTAATGACATTTGTCGAGTGGCAGATCTTAACTCGACAAATGTCATTACATCACTTATGTAGACATTGGGAAATGTATACATTTTT
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The complete Sanger sequence of an F2 male mouse hemizygous for the hs121 enhancer allele with 5% of base pairs mutated that was active in the transgenic reporter assays:

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The complete Sanger sequence of an F1 male mouse hemizygous for the hs121 enhancer allele with 5% of base pairs mutated that was inactive in the transgenic reporter assays:

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AGATGCAGATAAAAGCTCCCACTTGCTTCCCCAATGAGAGCTTAATCAATAGCTTATGGAAGGTCTTCTAGGAAACAGAACTGGATAAGCTGTCACAGGCCACATCATAGGGGCTGGGAGGGCAAGAAGTCATT
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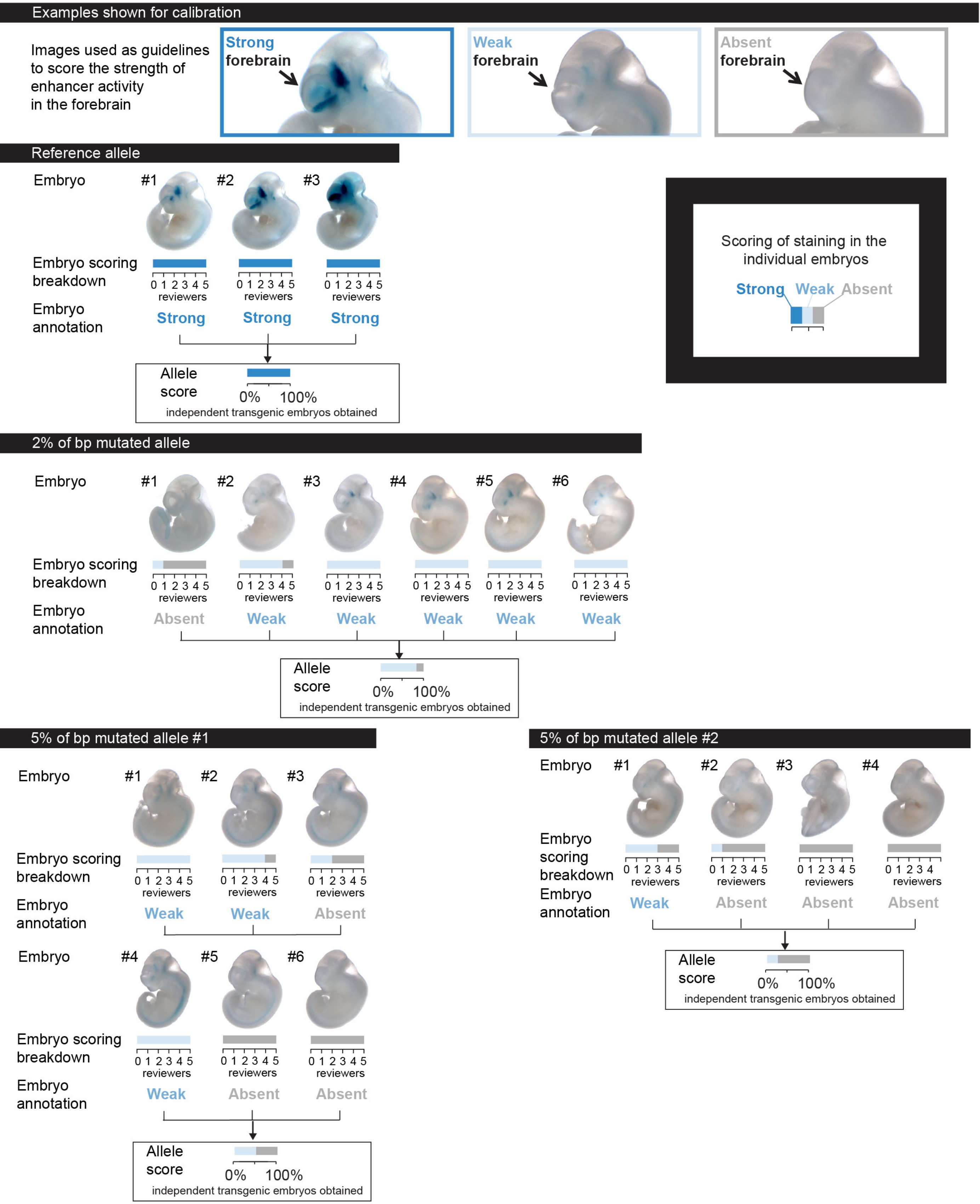
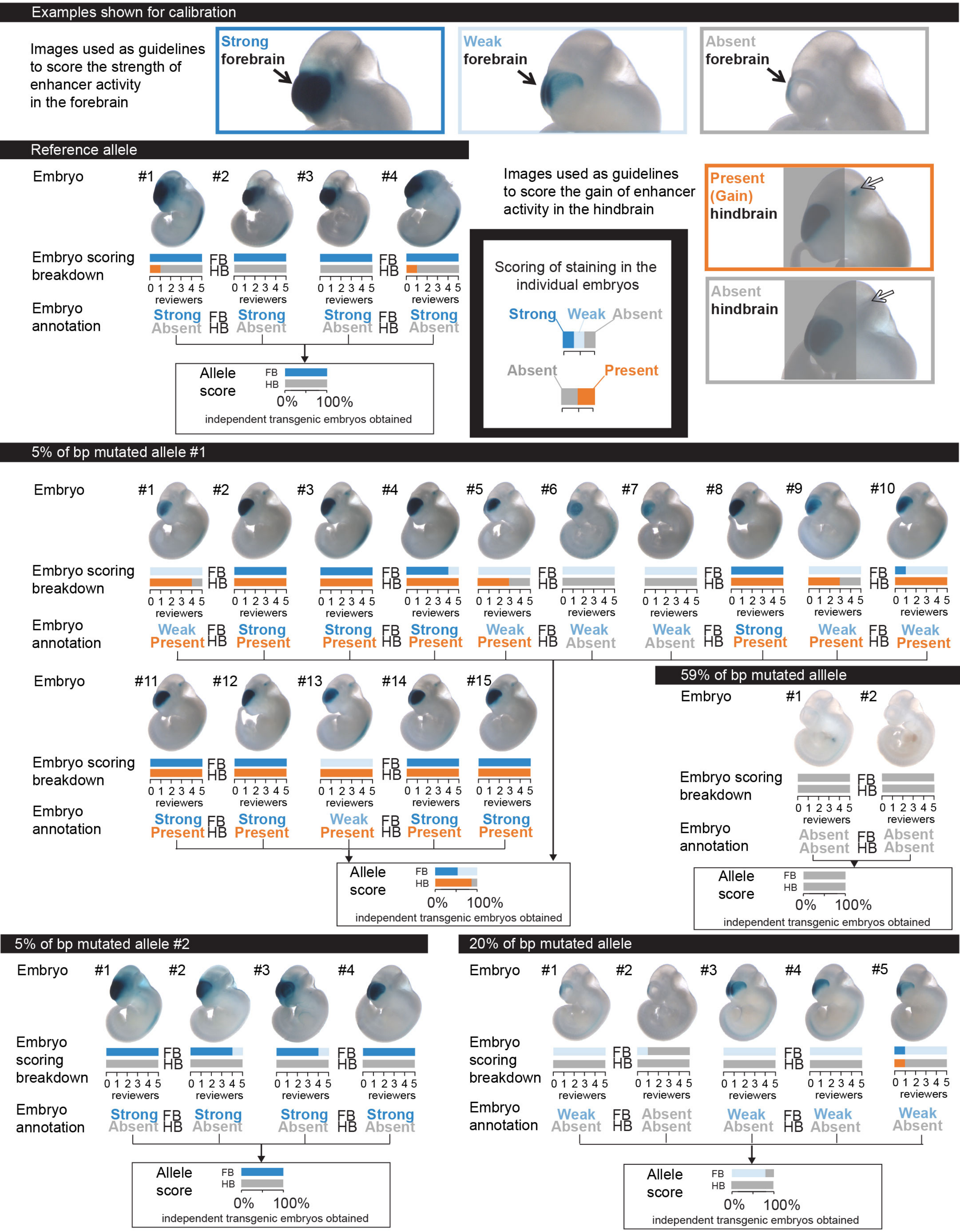
Supplementary Figure 1 | Images and scoring results of all transgenic embryos.

Locus: hs110 - uc206

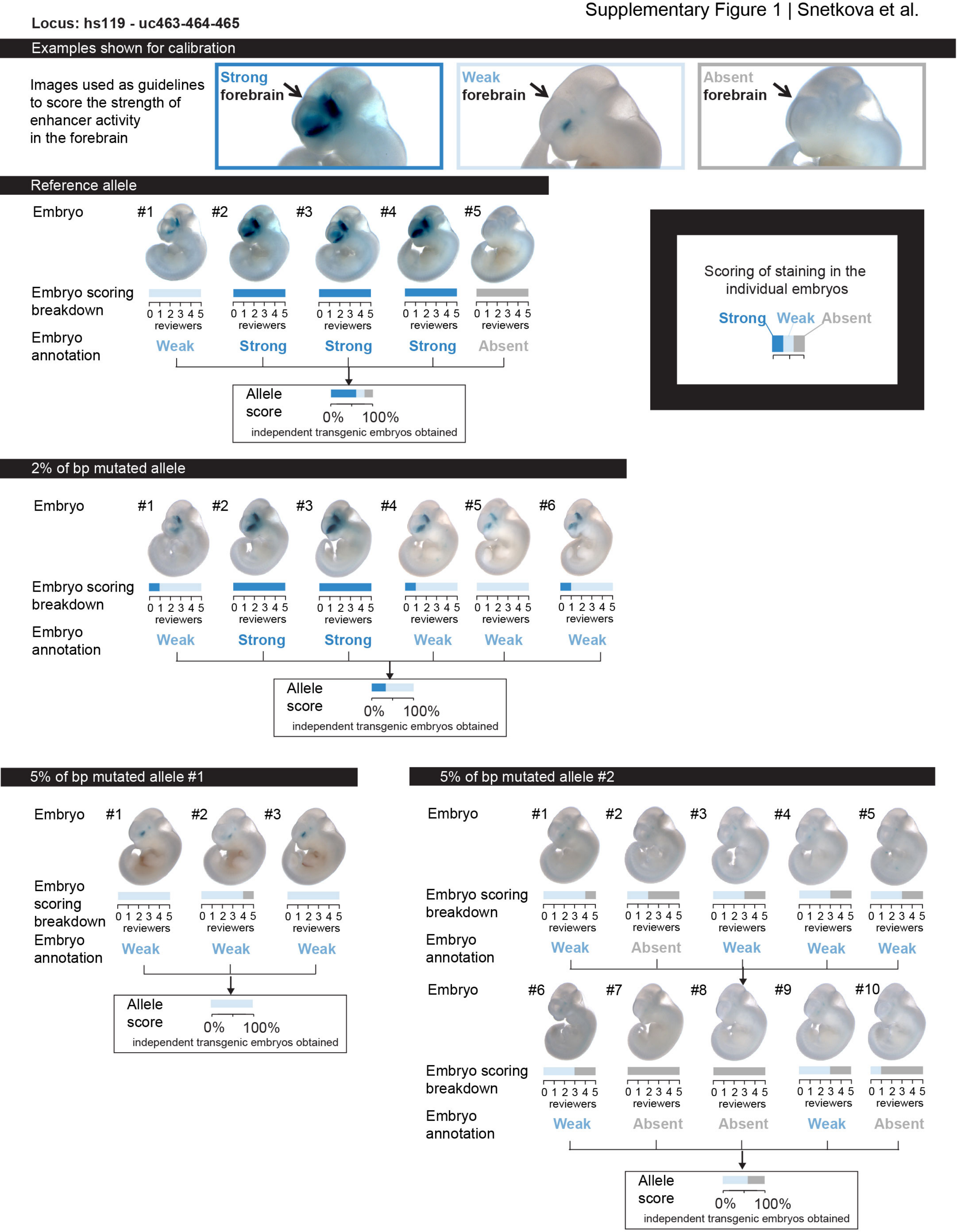


Supplementary Figure 1 | Snetkova et al.

Locus: hs111 - uc215

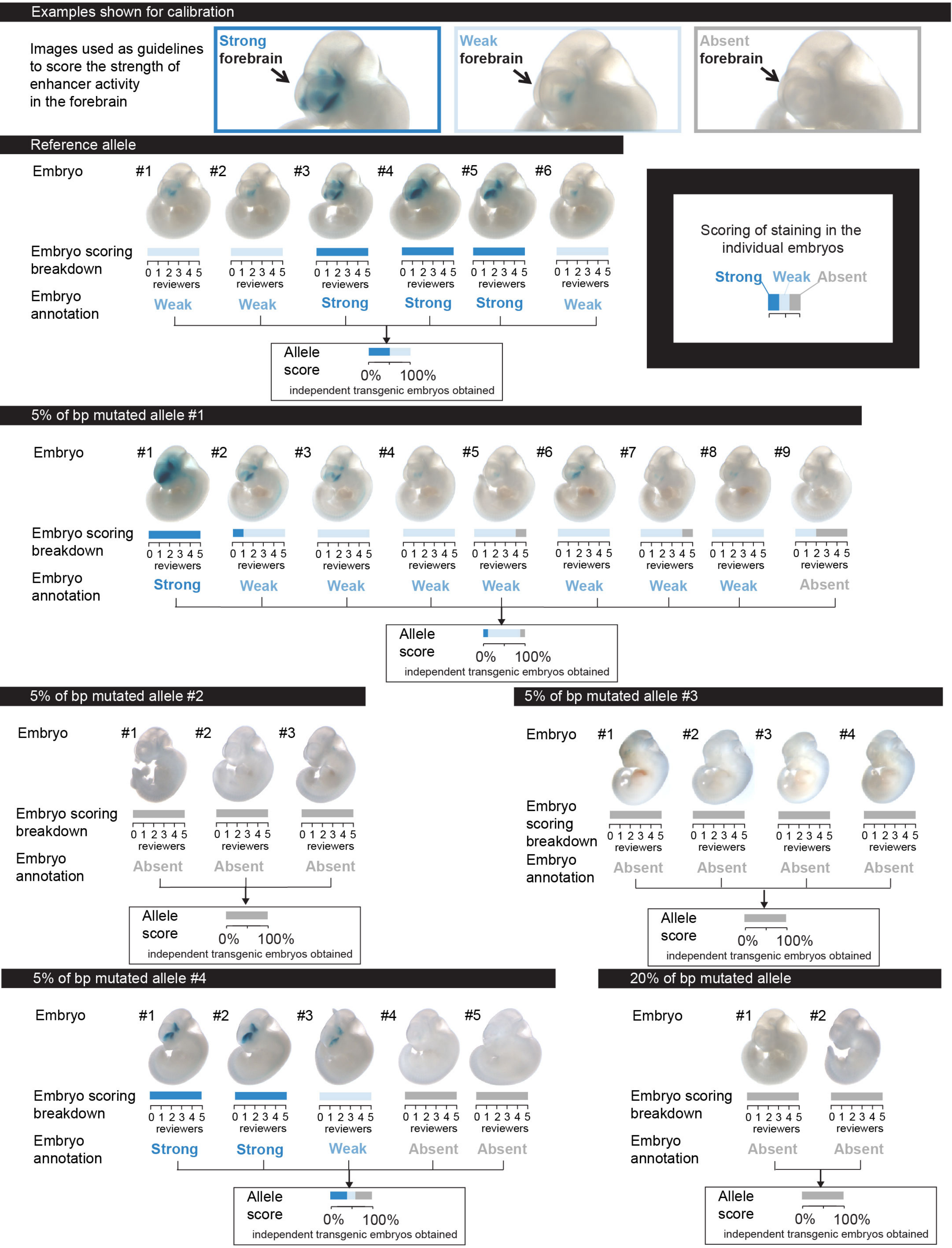


Supplementary Figure 1 | Snetkova et al.



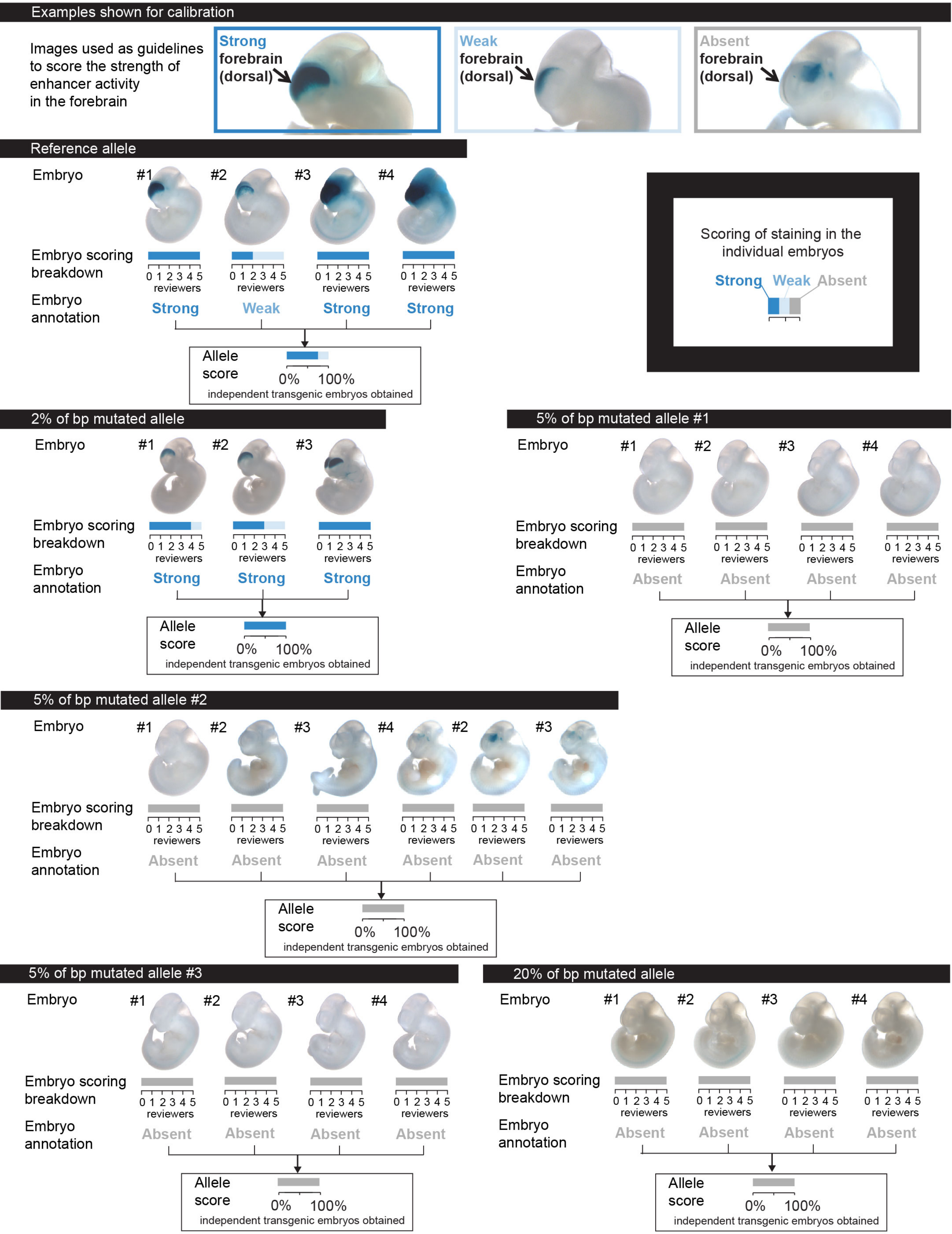
Locus: hs121 - uc467

Supplementary Figure 1 | Snetkova et al.



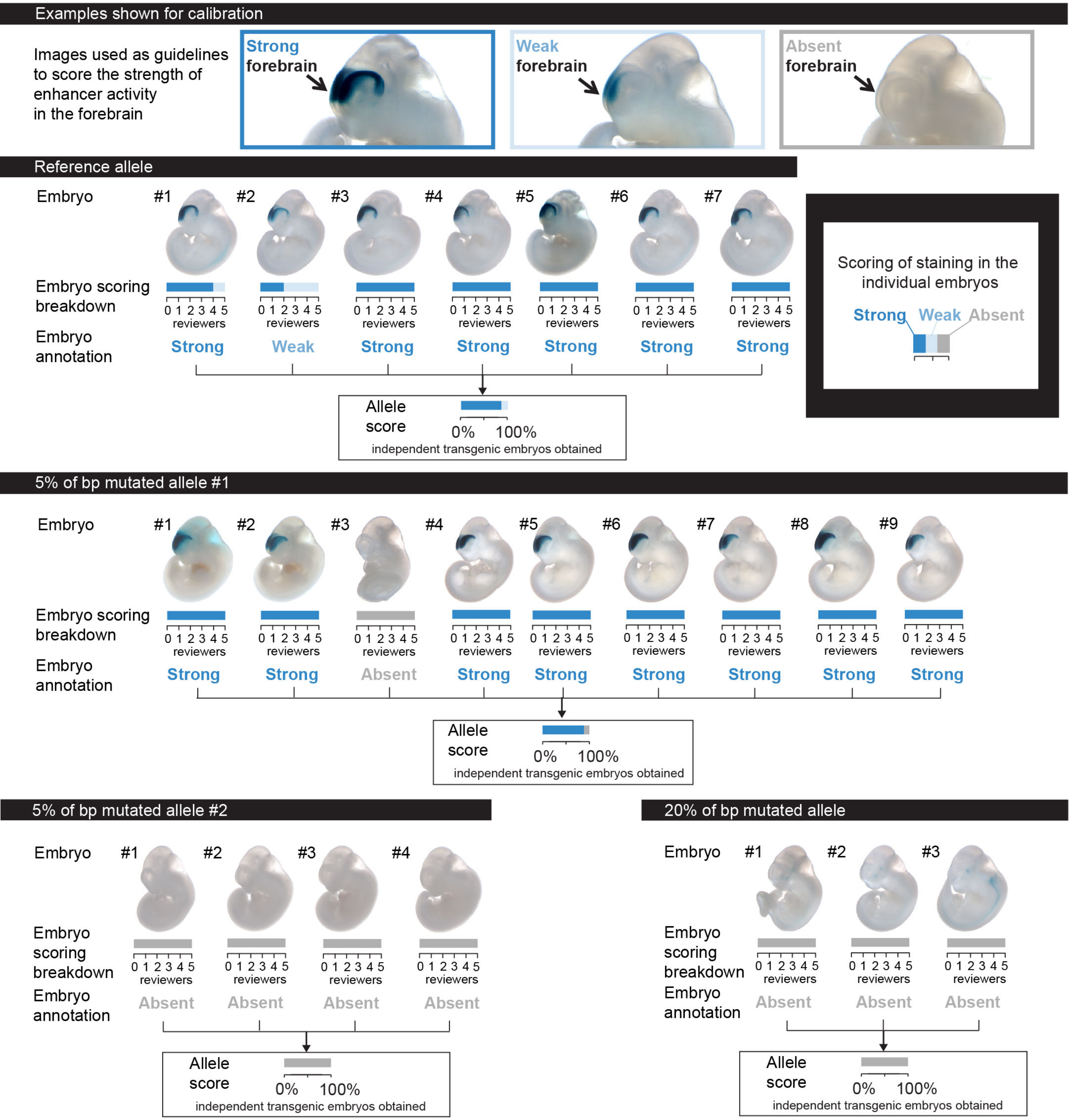
Locus: hs122 - uc468-469

Supplementary Figure 1 | Snetkova et al.



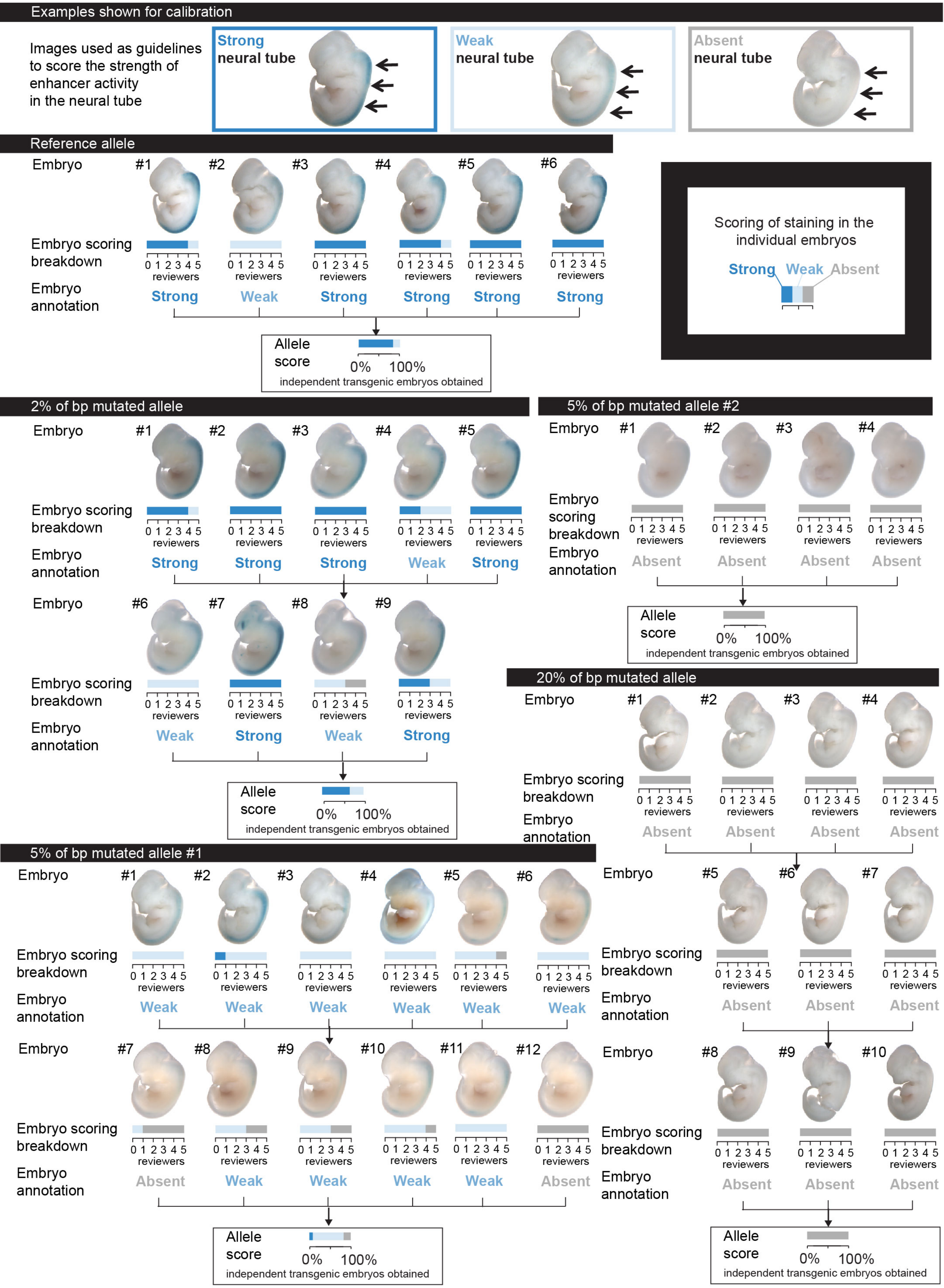
Supplementary Figure 1 | Snetkova et al.

Locus: hs123 - uc470



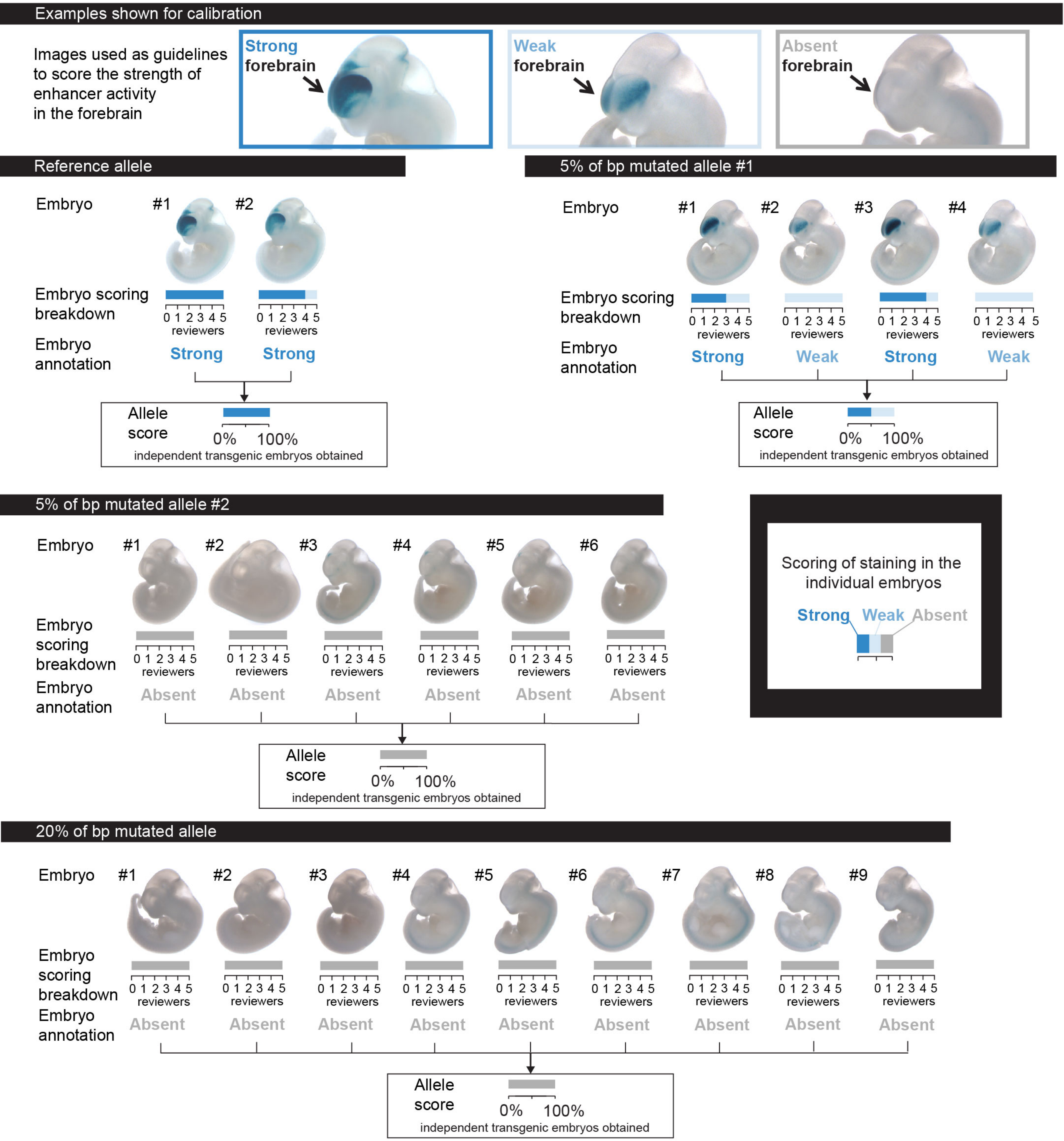
Supplementary Figure 1 | Snetkova et al.

Locus: hs124 - uc396

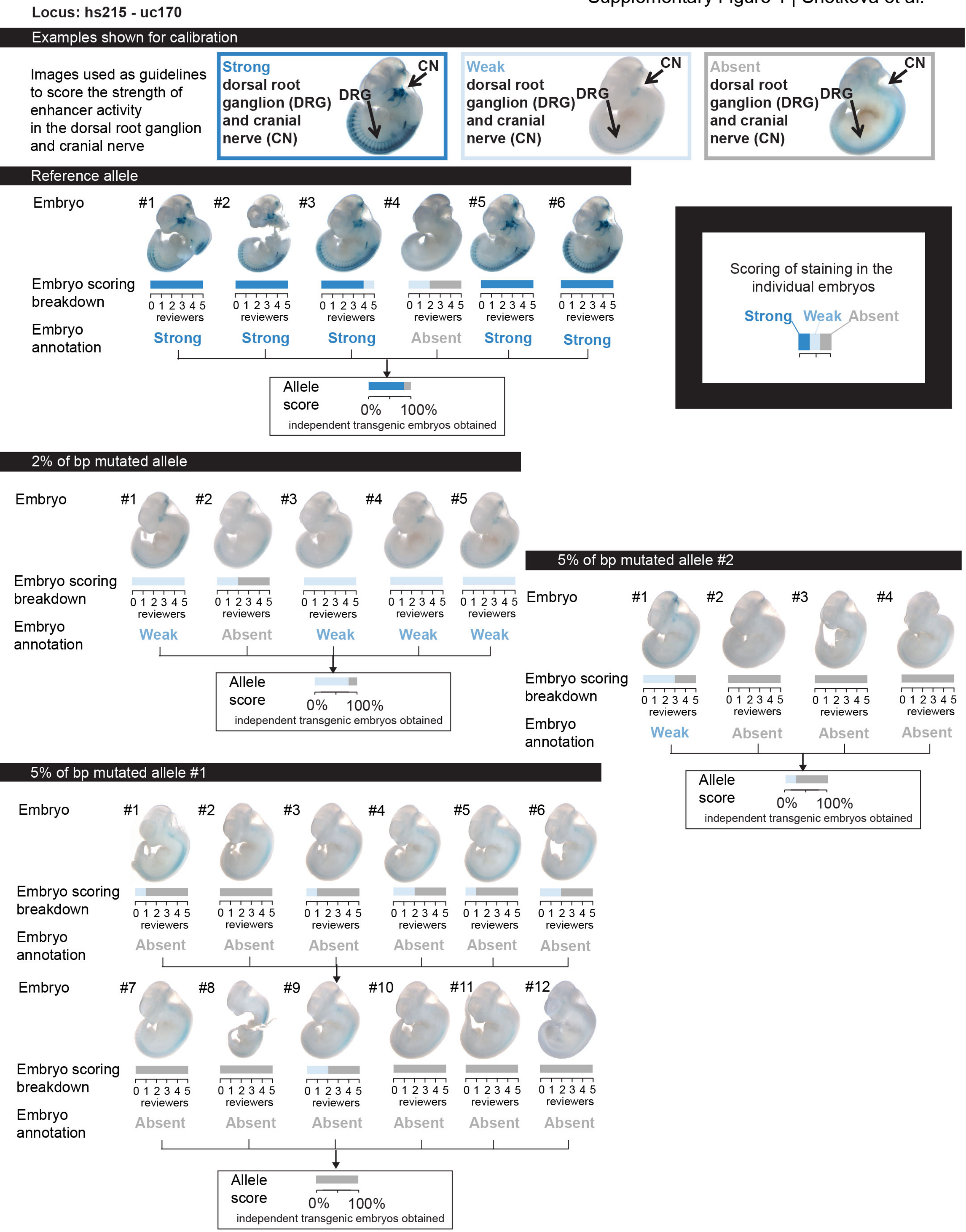


Supplementary Figure 1 | Snetkova et al.

Locus: hs200 - uc025



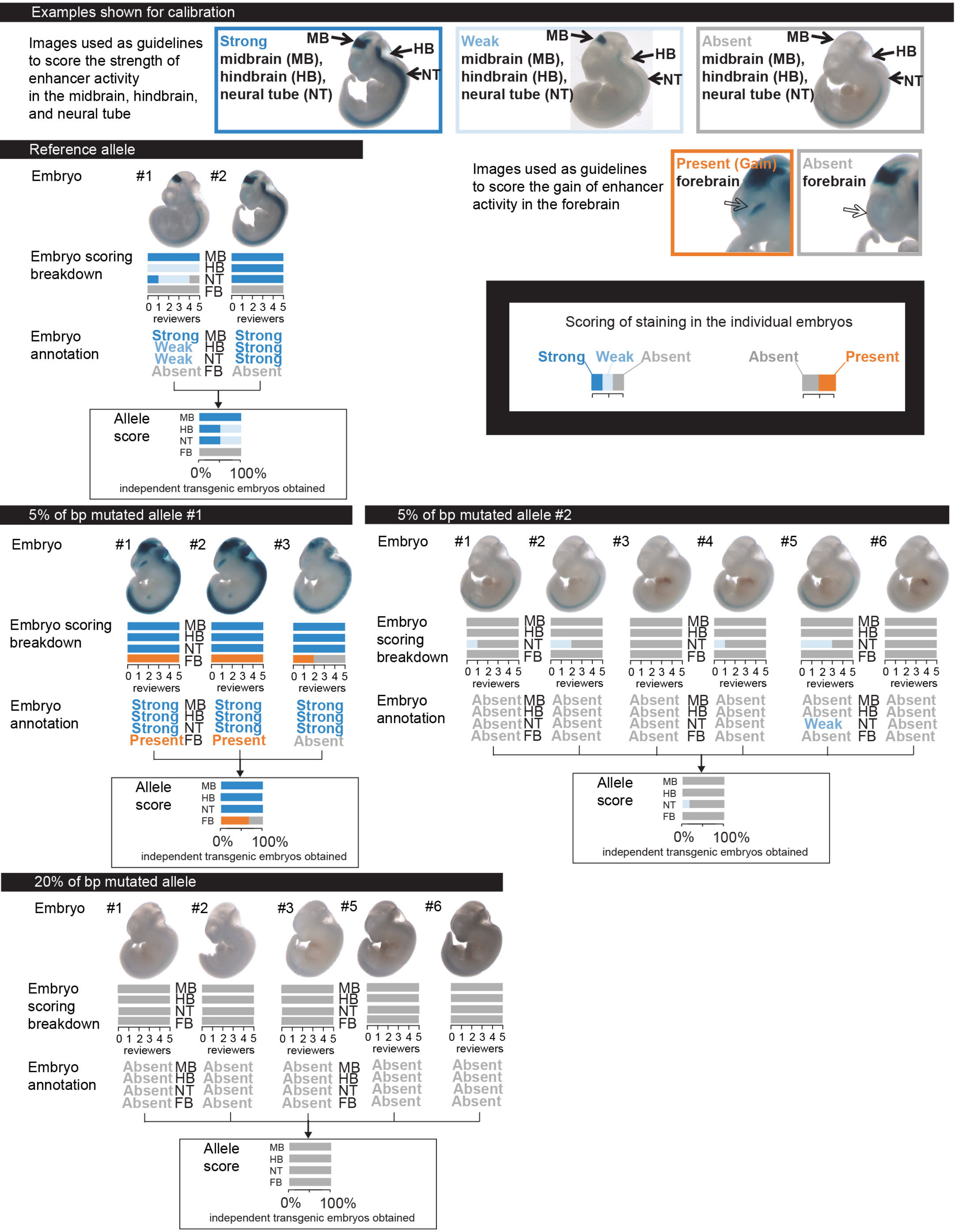
Supplementary Figure 1 | Snetkova et al.



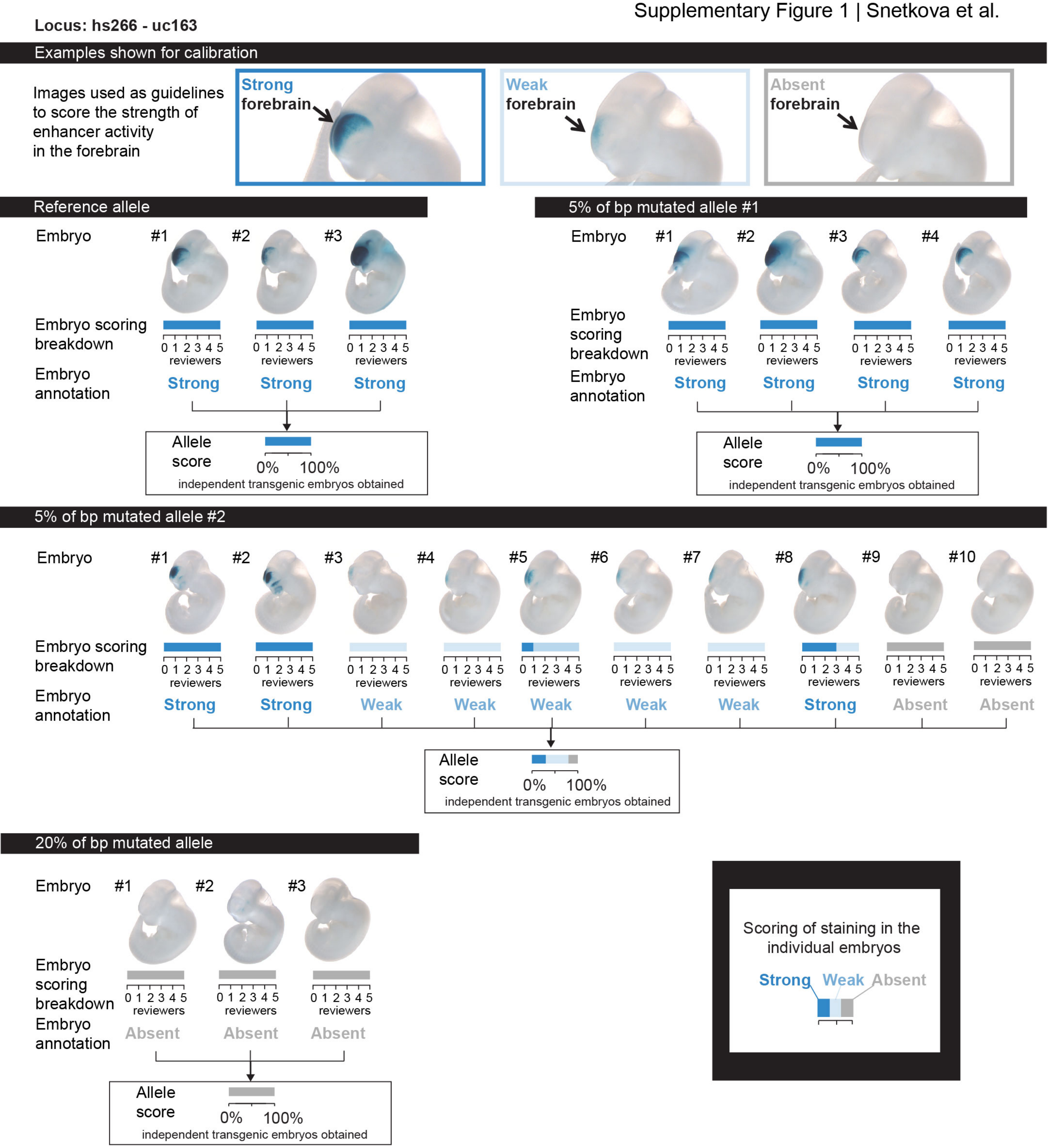
Supplementary Figure 1 | Snetkova et al.

Supplementary Figure 1 | Snetkova et al.

Locus: hs260 - uc145

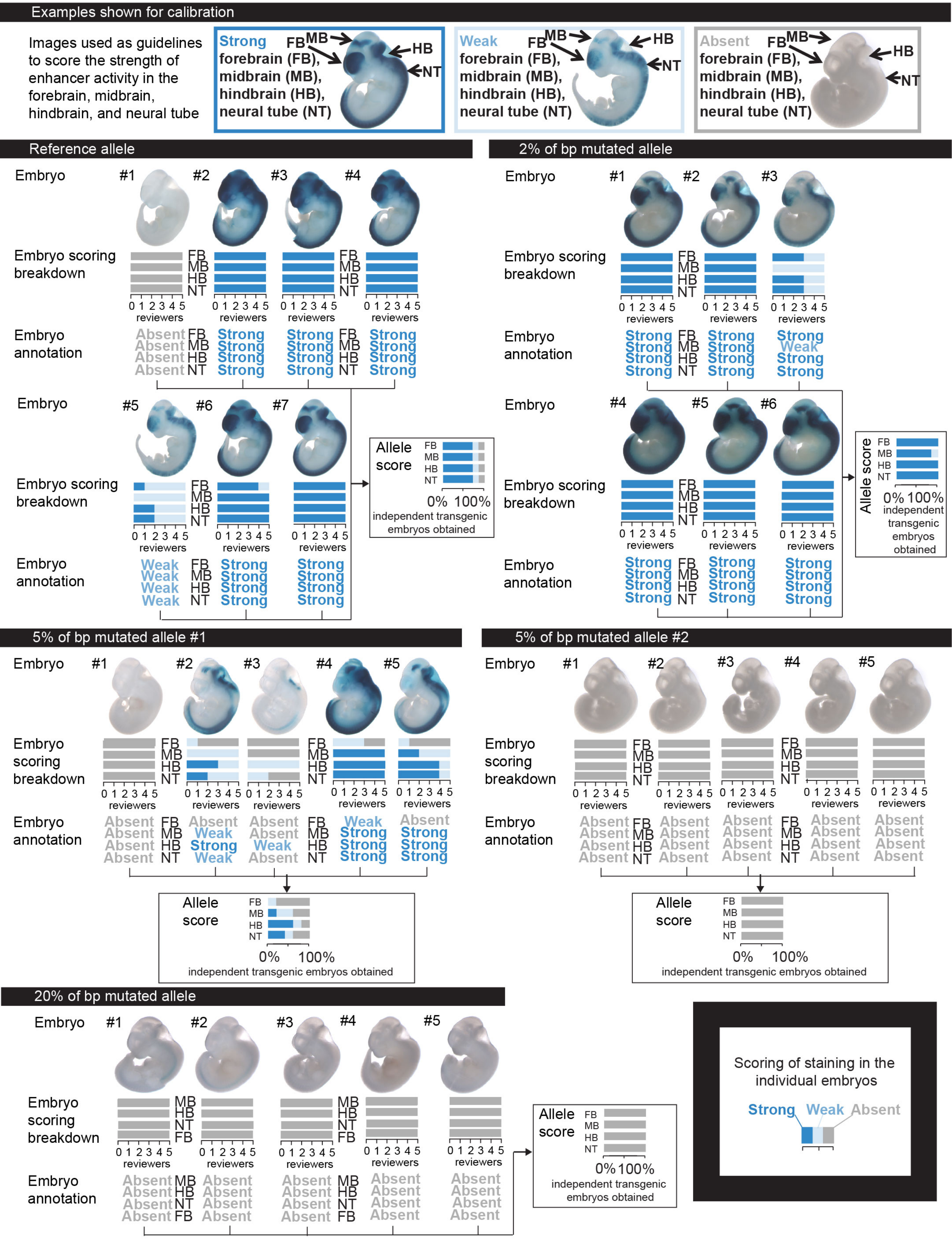


Supplementary Figure 1 | Snetkova et al.



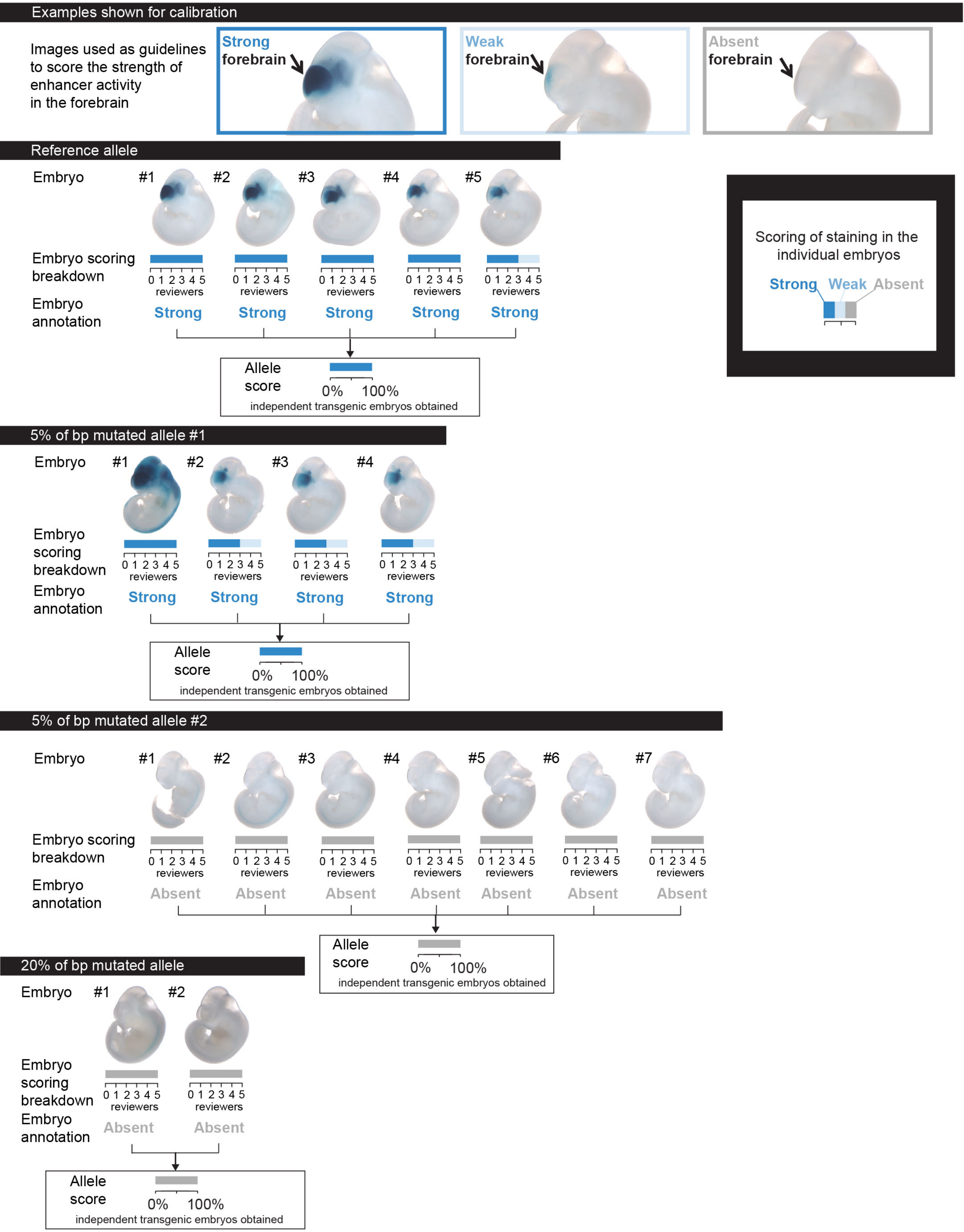
Locus: hs267 - uc164

Supplementary Figure 1 | Snetkova et al.



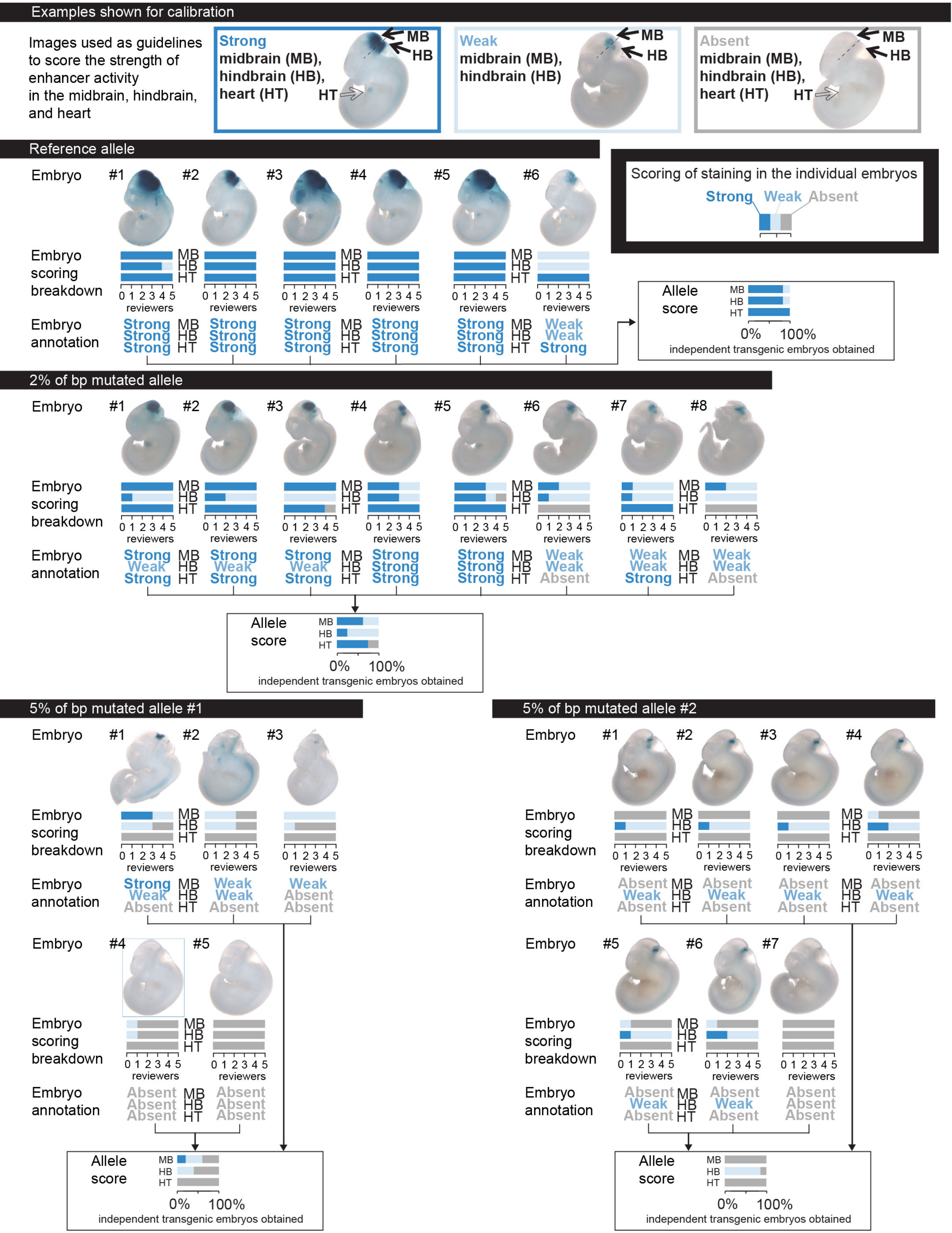
Supplementary Figure 1 | Snetkova et al.

Locus: hs271 - uc170

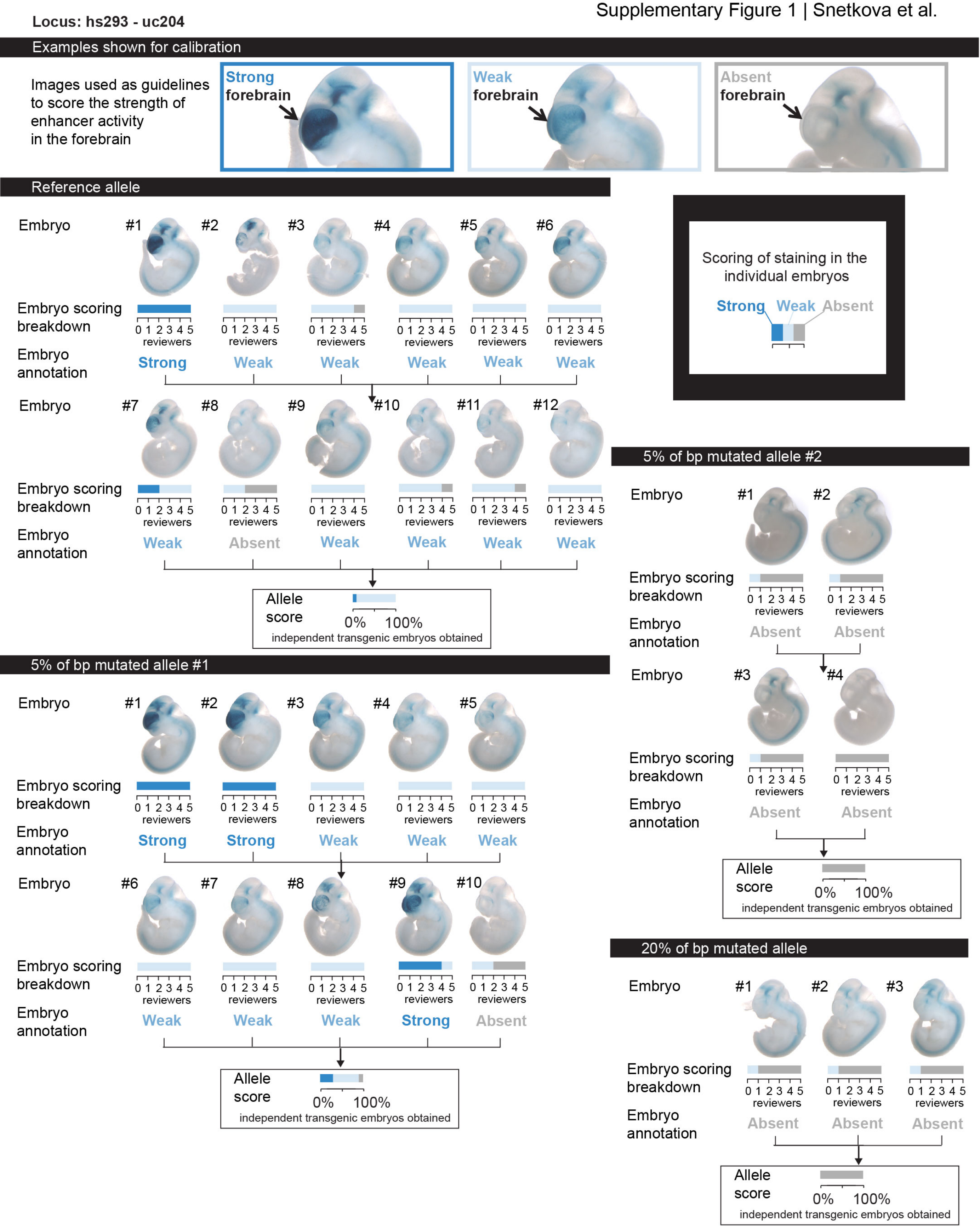


Supplementary Figure 1 | Snetkova et al.

Locus: hs282 - uc196

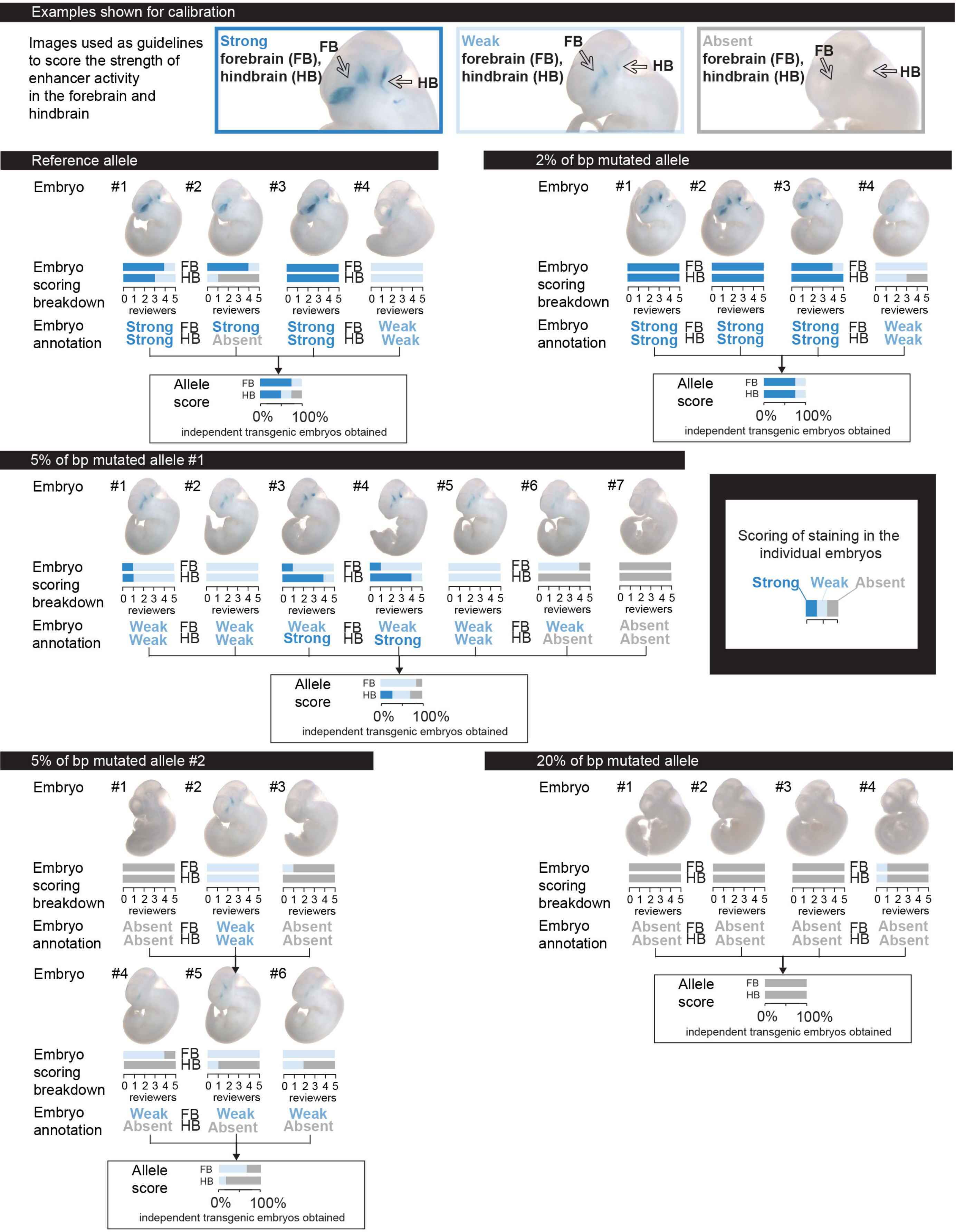


Supplementary Figure 1 | Snetkova et al.



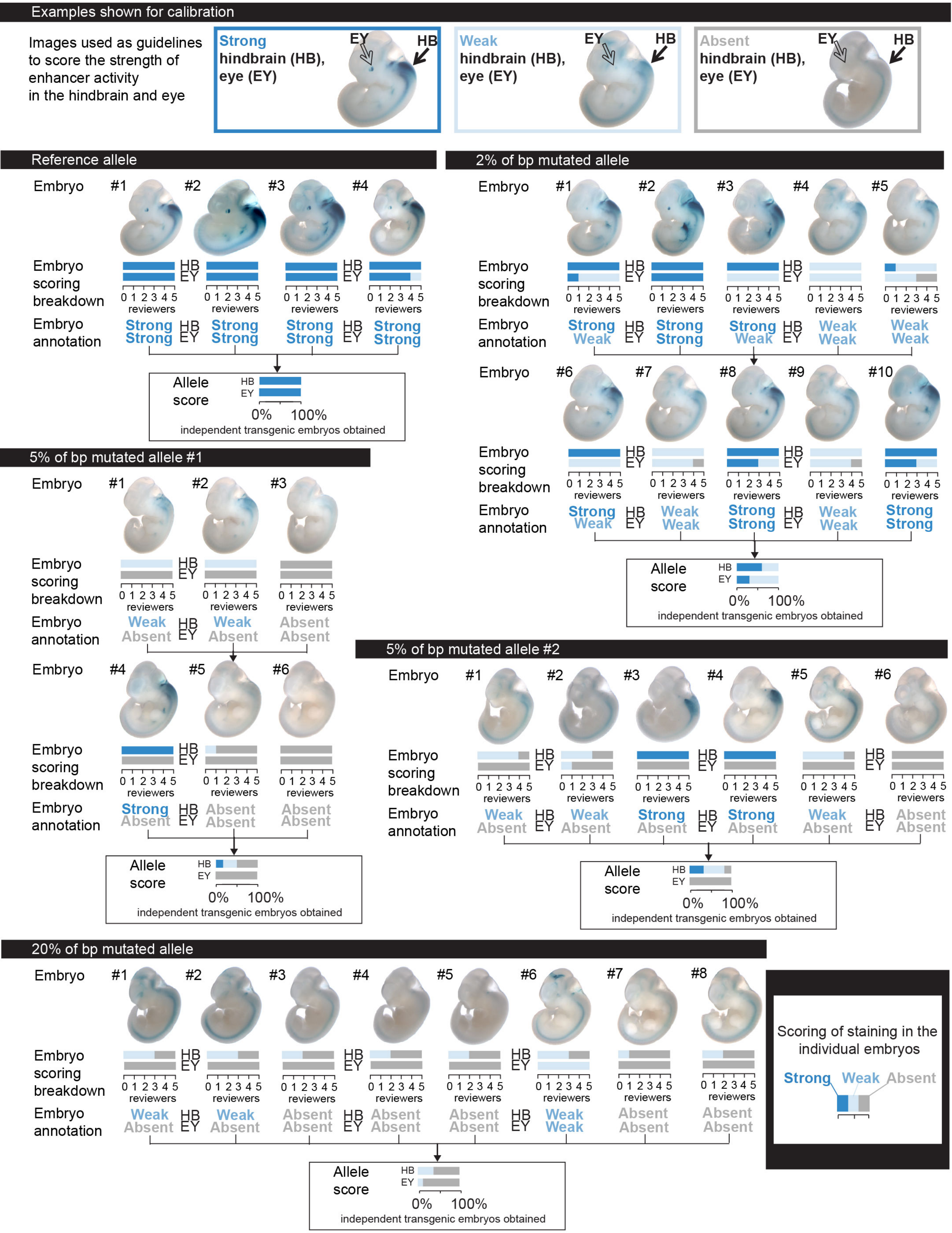
Supplementary Figure 1 | Snetkova et al.

Locus: hs312 - uc260



Supplementary Figure 1 | Snetkova et al.

Locus: hs320 - uc288

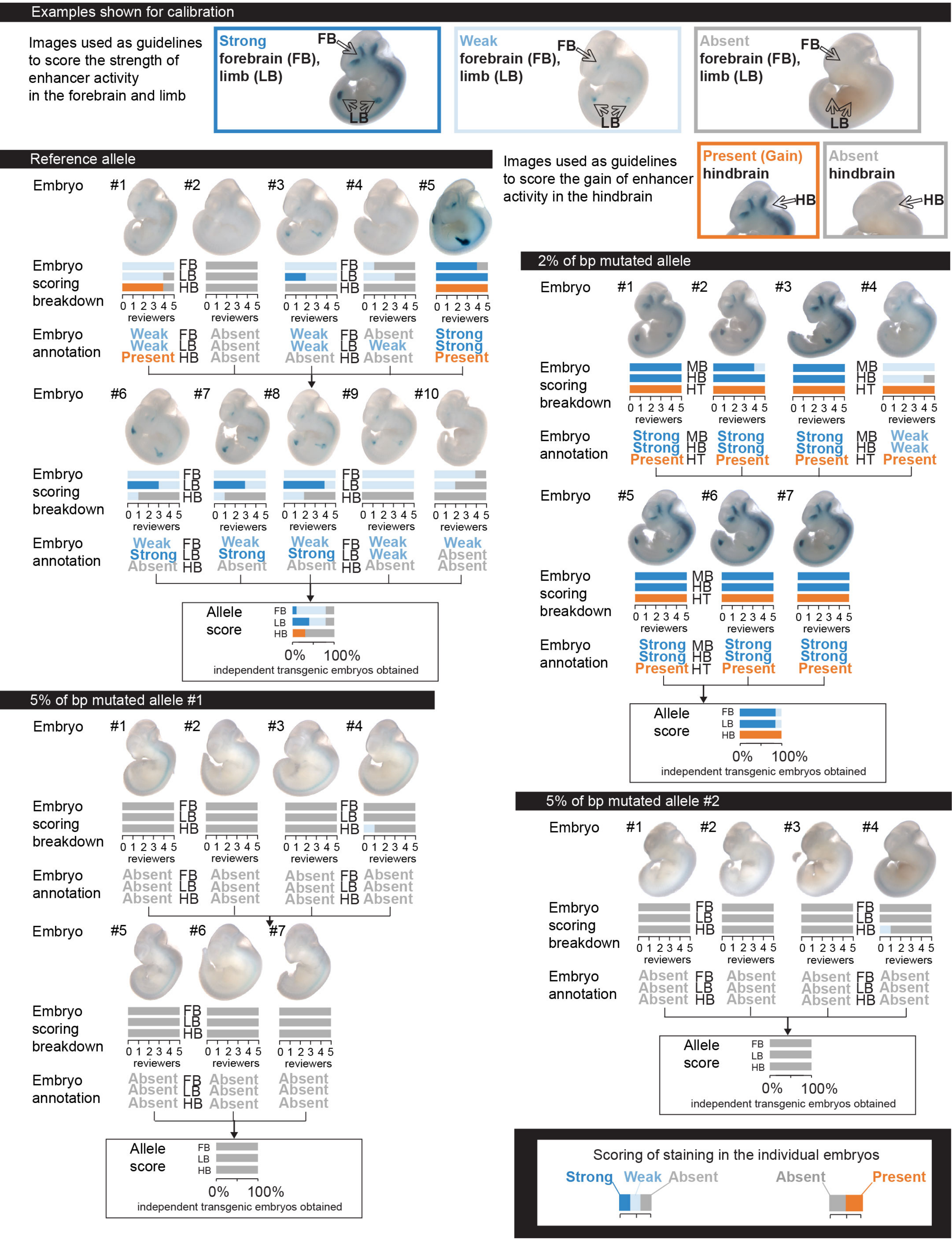


Supplementary Figure 1 | Snetkova et al.

Locus: hs327 - uc031

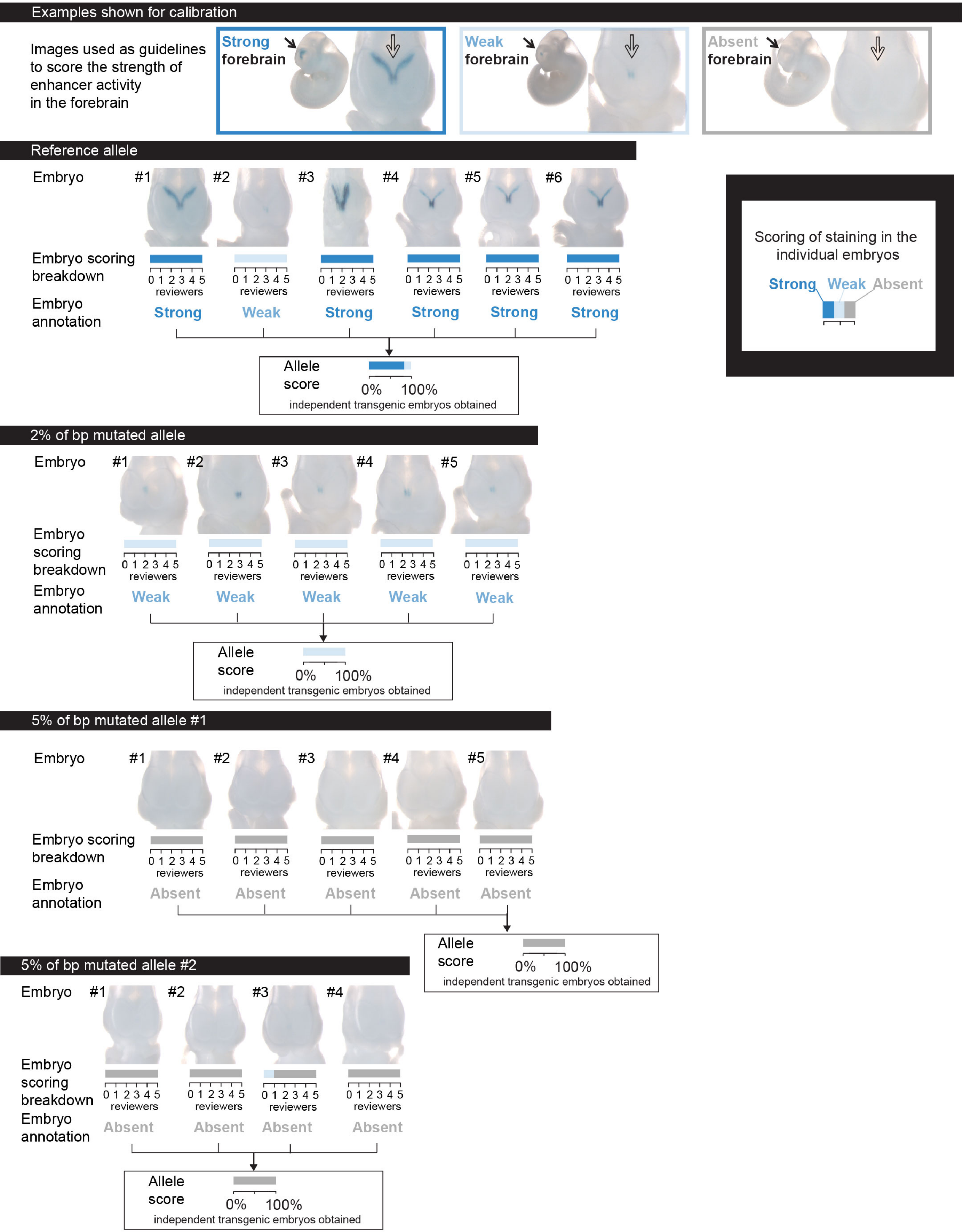
Locus: hs388 - uc047

Supplementary Figure 1 | Snetkova et al.



Supplementary Figure 1 | Snetkova et al.

Locus: hs411 - uc082

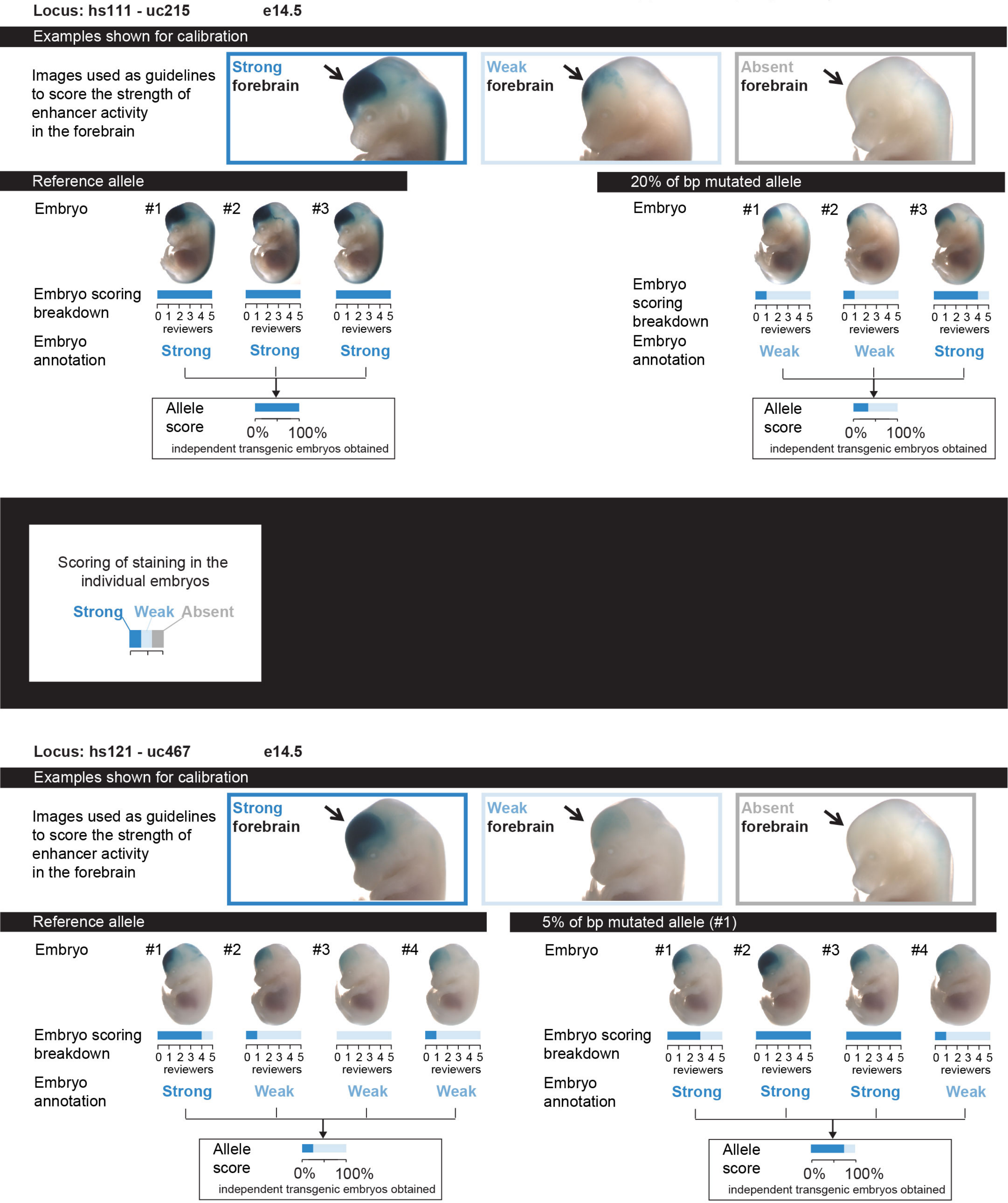


Supplementary Figure 1 | Snetkova et al.

Locus: hs426 - uc476

Supplementary Figure 1 | Snetkova et al.

Supplementary Figure 1 | Snetkova et al.

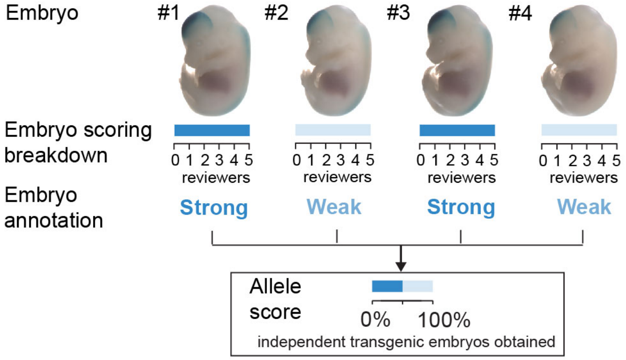


Locus: hs123 - uc470 **e14.5**

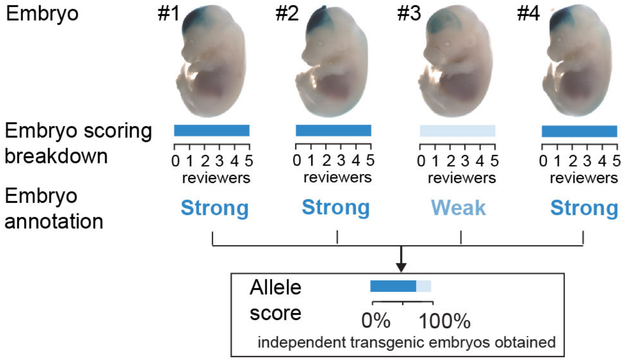
Examples shown for calibration

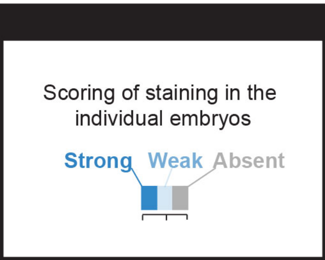
Images used as guidelines to score the strength of enhancer activity in the forebrain

Reference allele



5% of bp mutated allele (#1)

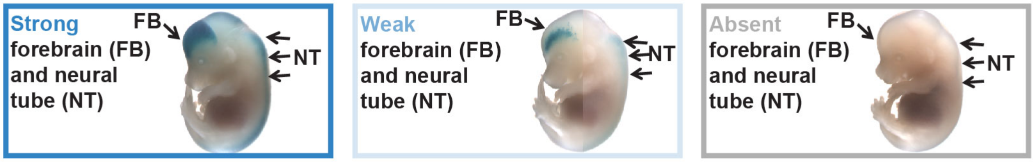




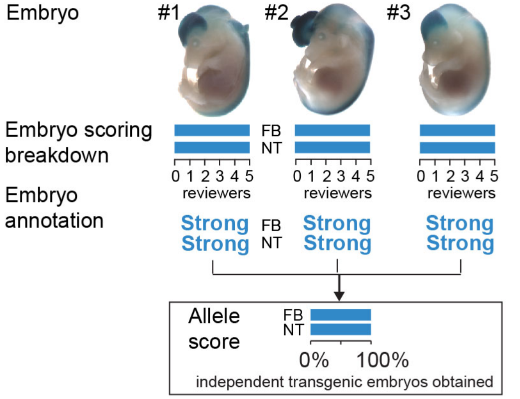
Locus: hs200 - uc025 **e14.5**

Examples shown for calibration

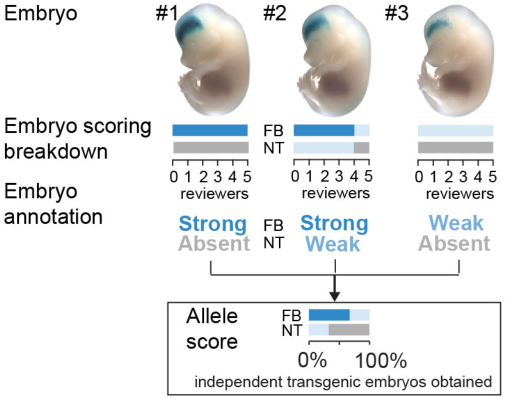
Images used as guidelines to score the strength of enhancer activity in the forebrain and neural tube



Reference allele



5% of bp mutated allele (#1)

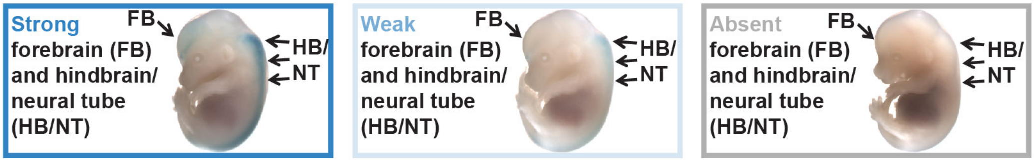


Supplementary Figure 1 | Snetkova et al.

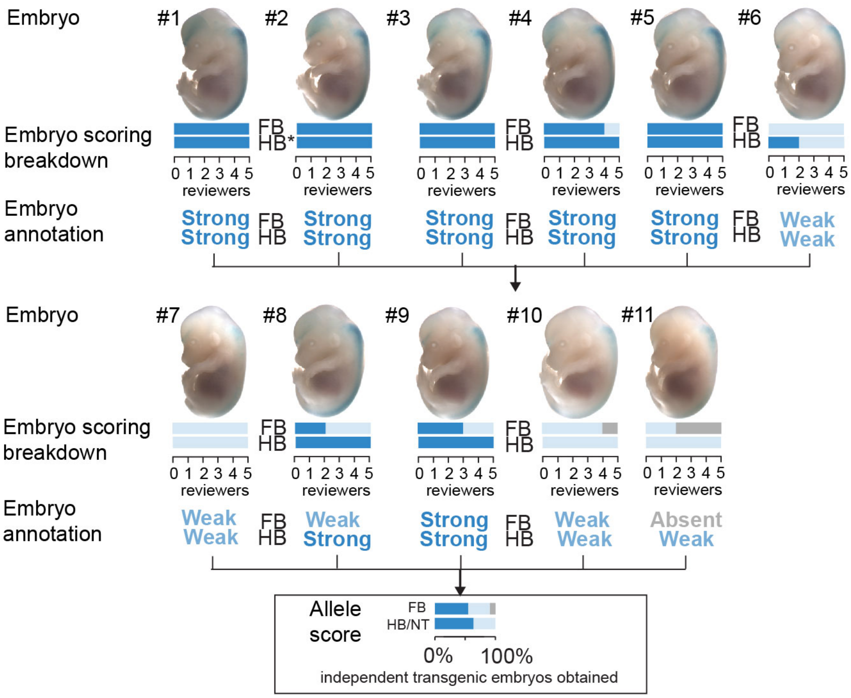
Locus: hs246 - uc108 **e14.5**

Examples shown for calibration

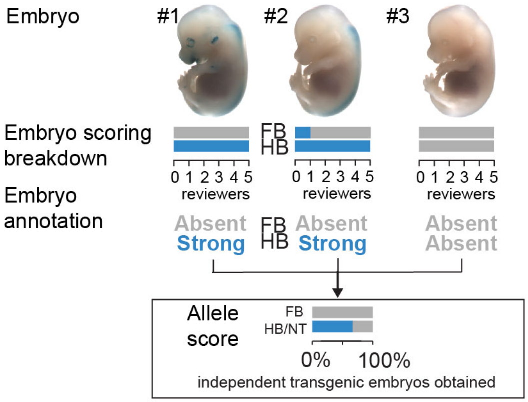
Images used as guidelines to score the strength of enhancer activity in the forebrain and hindbrain / neural tube

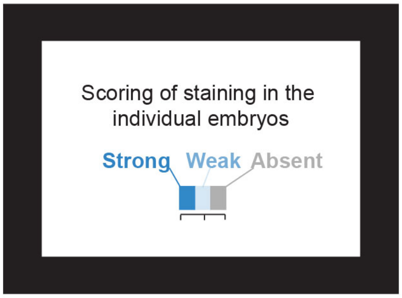


Reference allele



5% of bp mutated allele (#1)

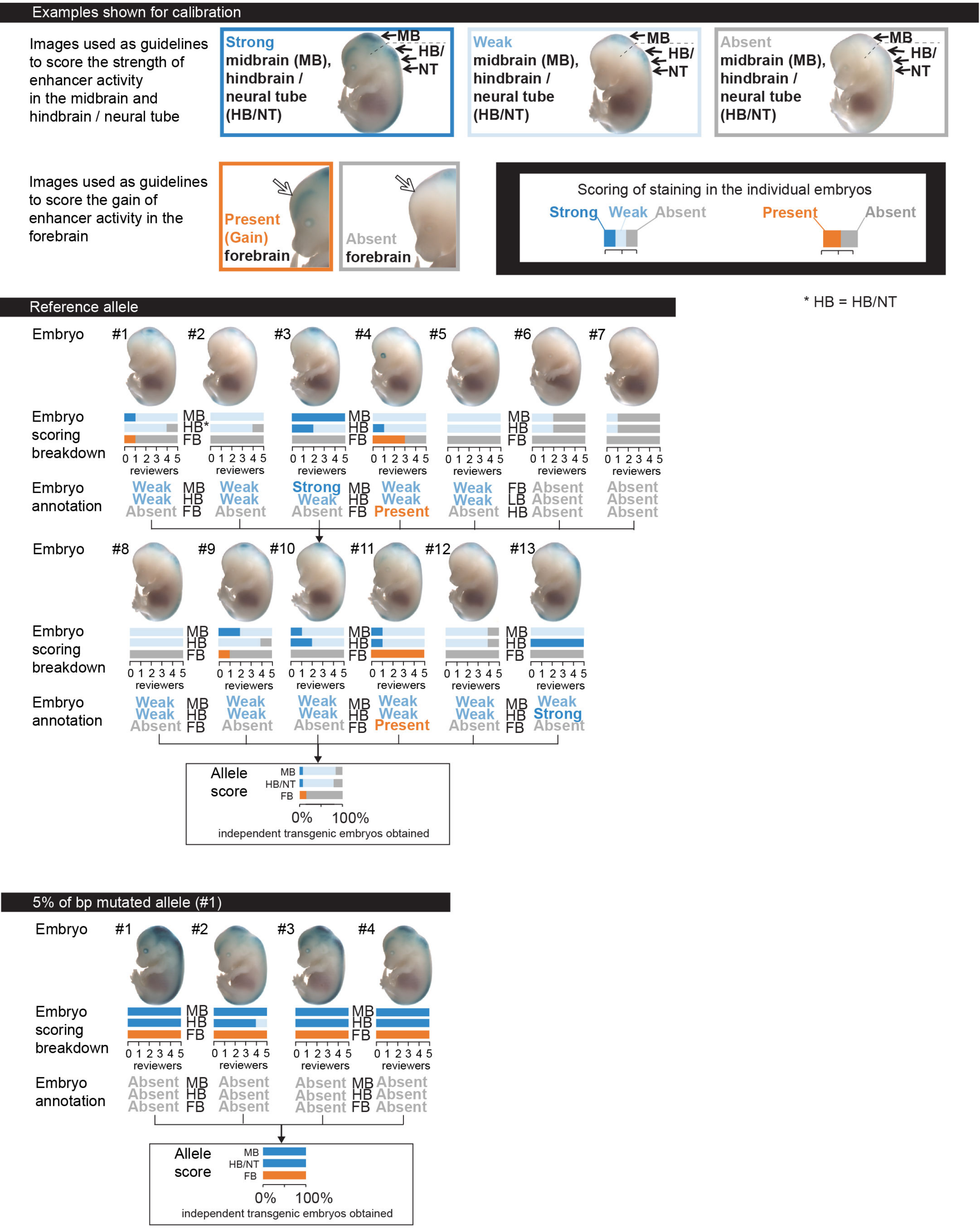




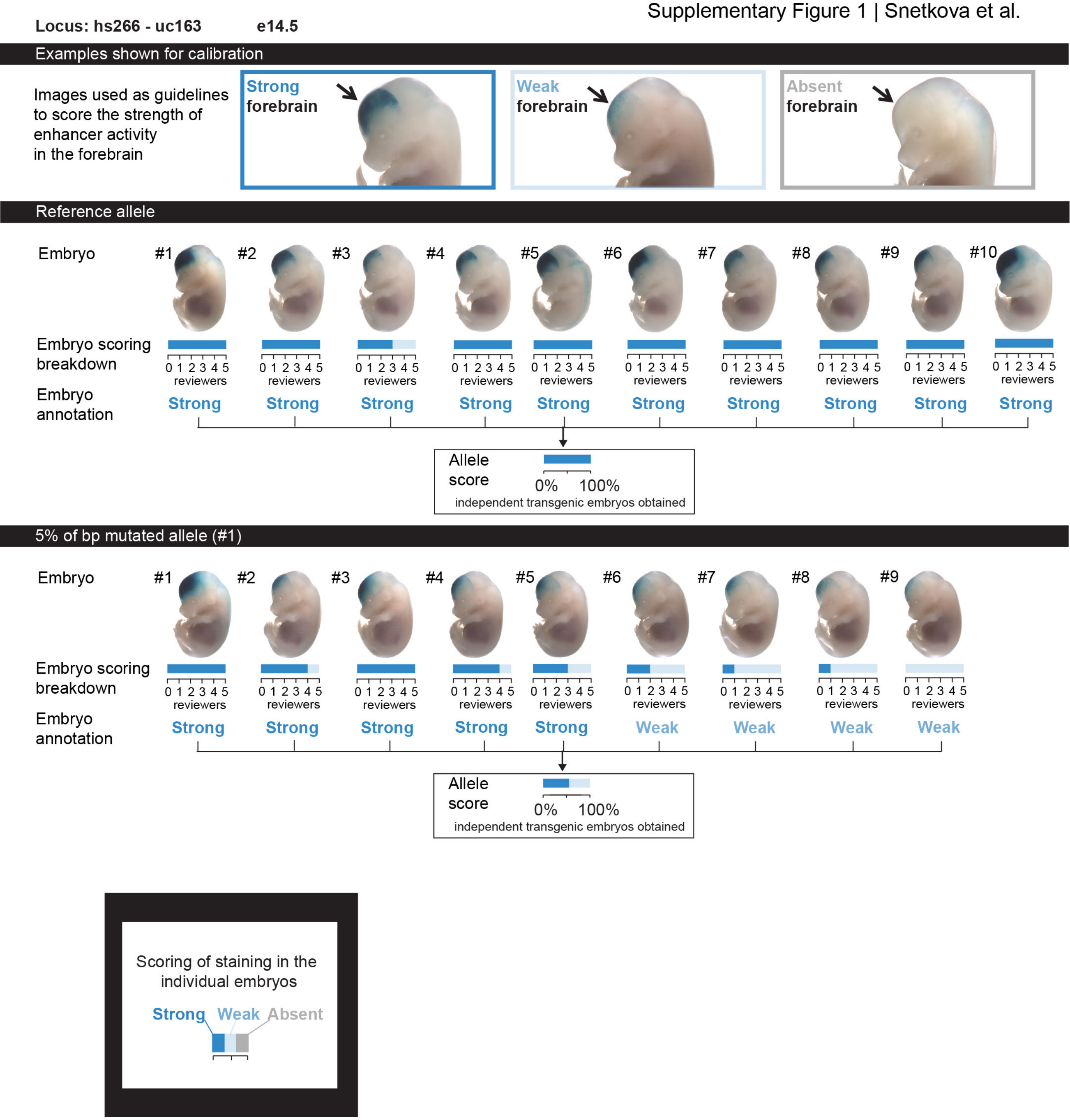
* HB = HB/NT

Supplementary Figure 1 | Snetkova et al.

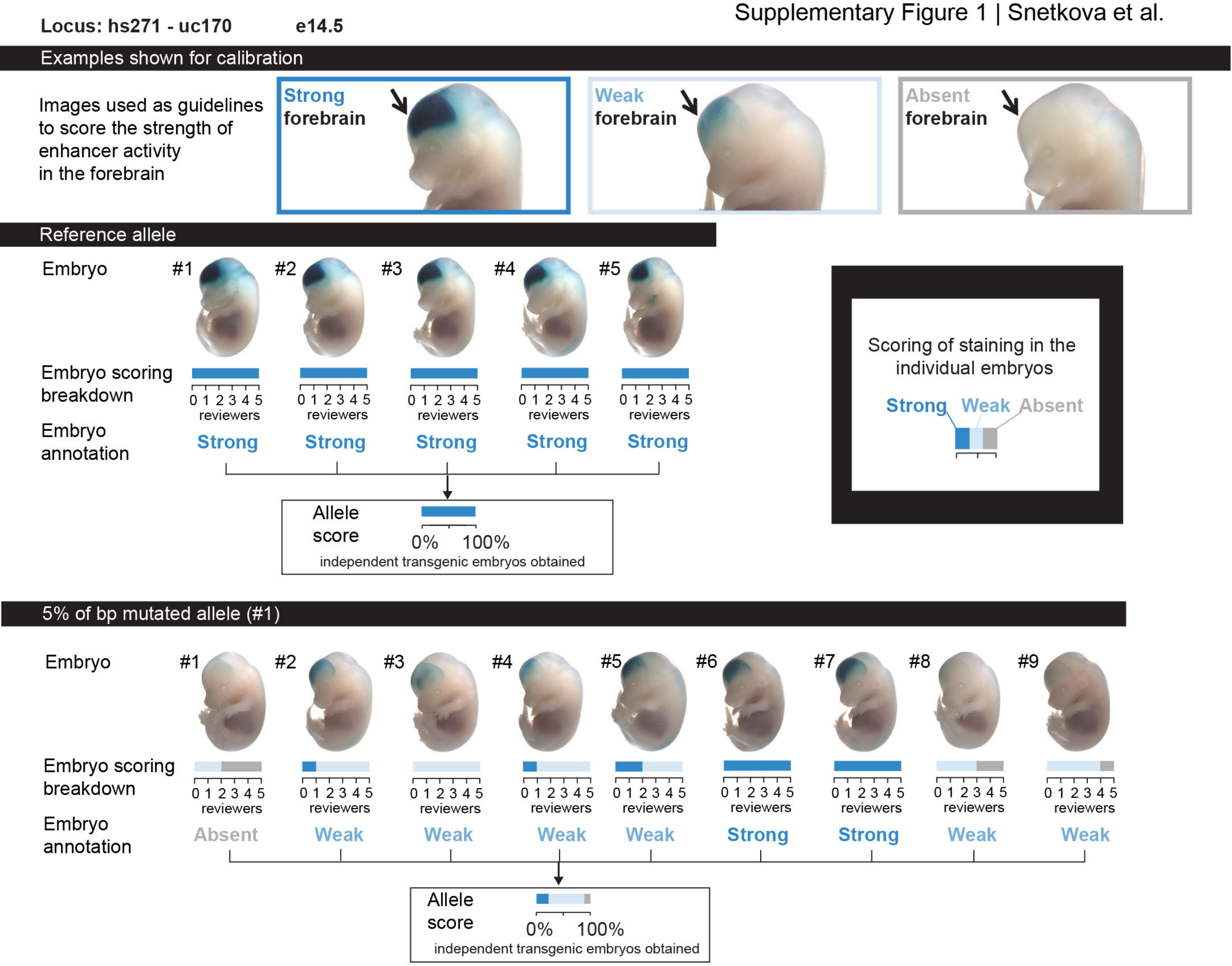
Locus: hs260 - uc145



Supplementary Figure 1 | Snetkova et al.



Supplementary Figure 1 | Snetkova et al.



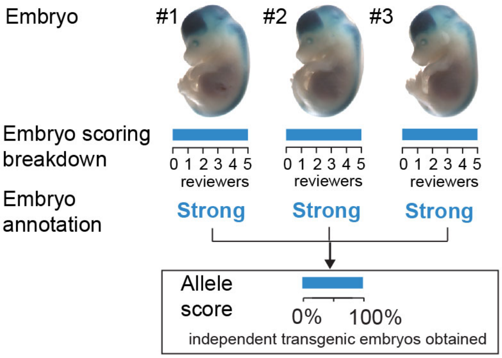
Supplementary Figure 1 | Snetkova et al.

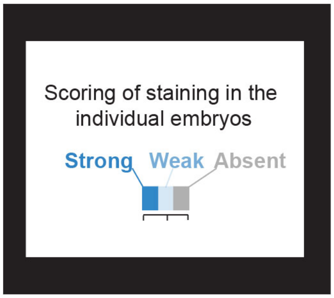
Locus: hs293 - uc204 **e14.5**

Examples shown for calibration

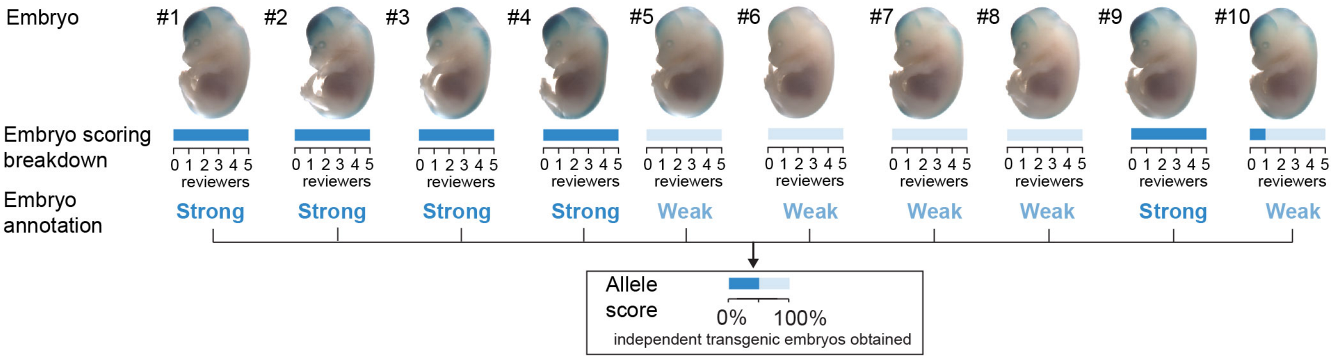
Images used as guidelines to score the strength of enhancer activity in the forebrain

Reference allele

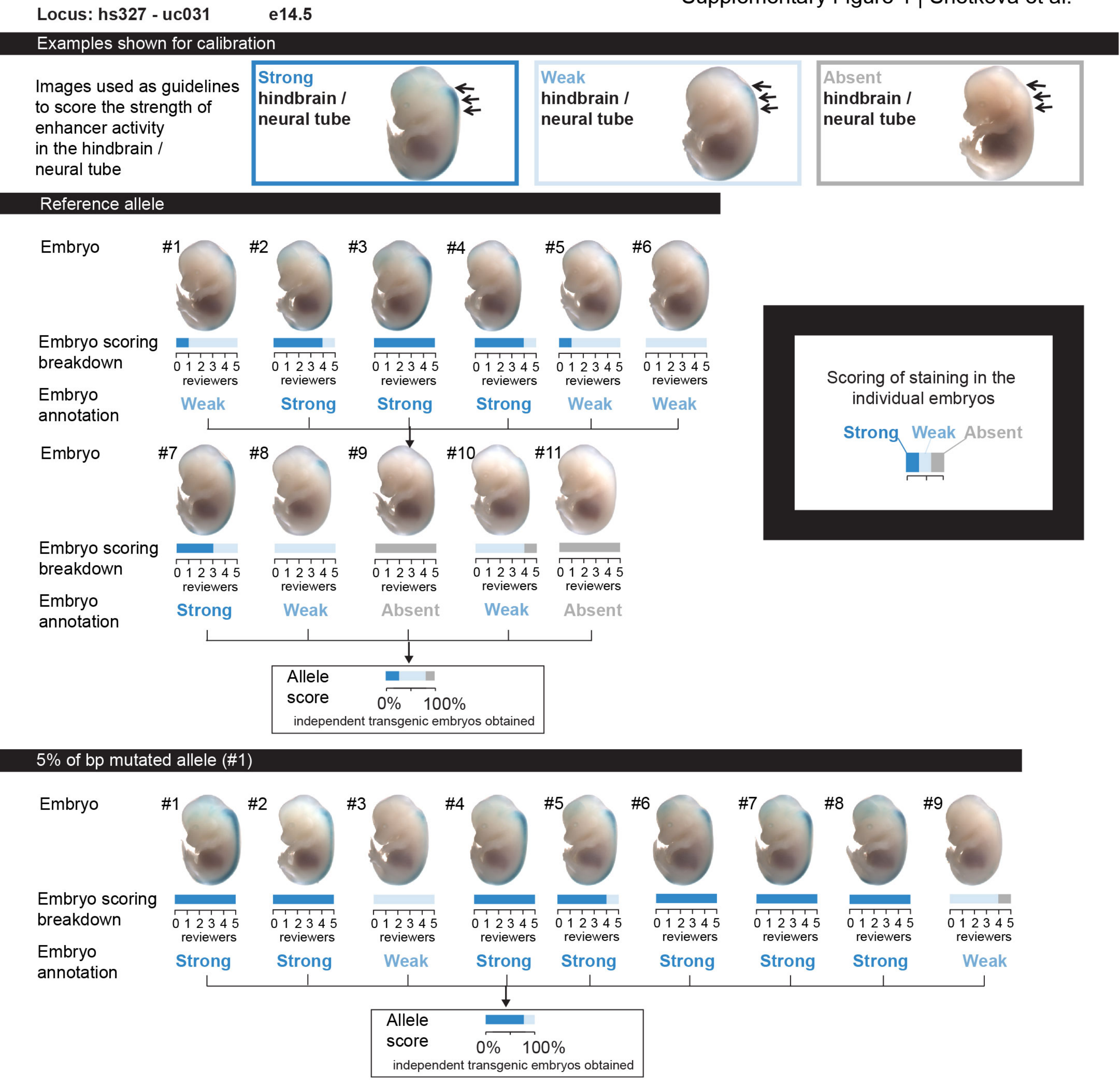




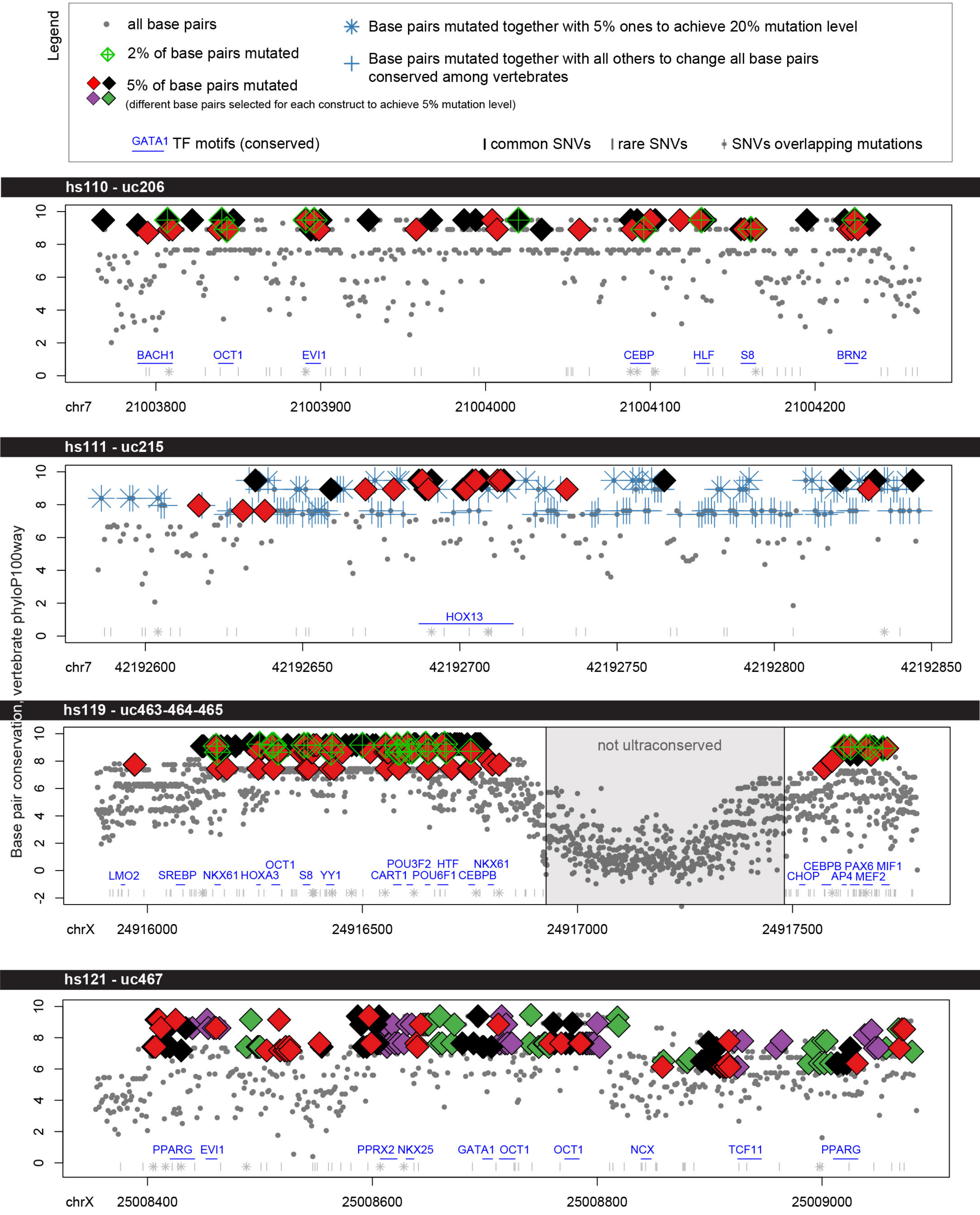
5% of bp mutated allele (#1)



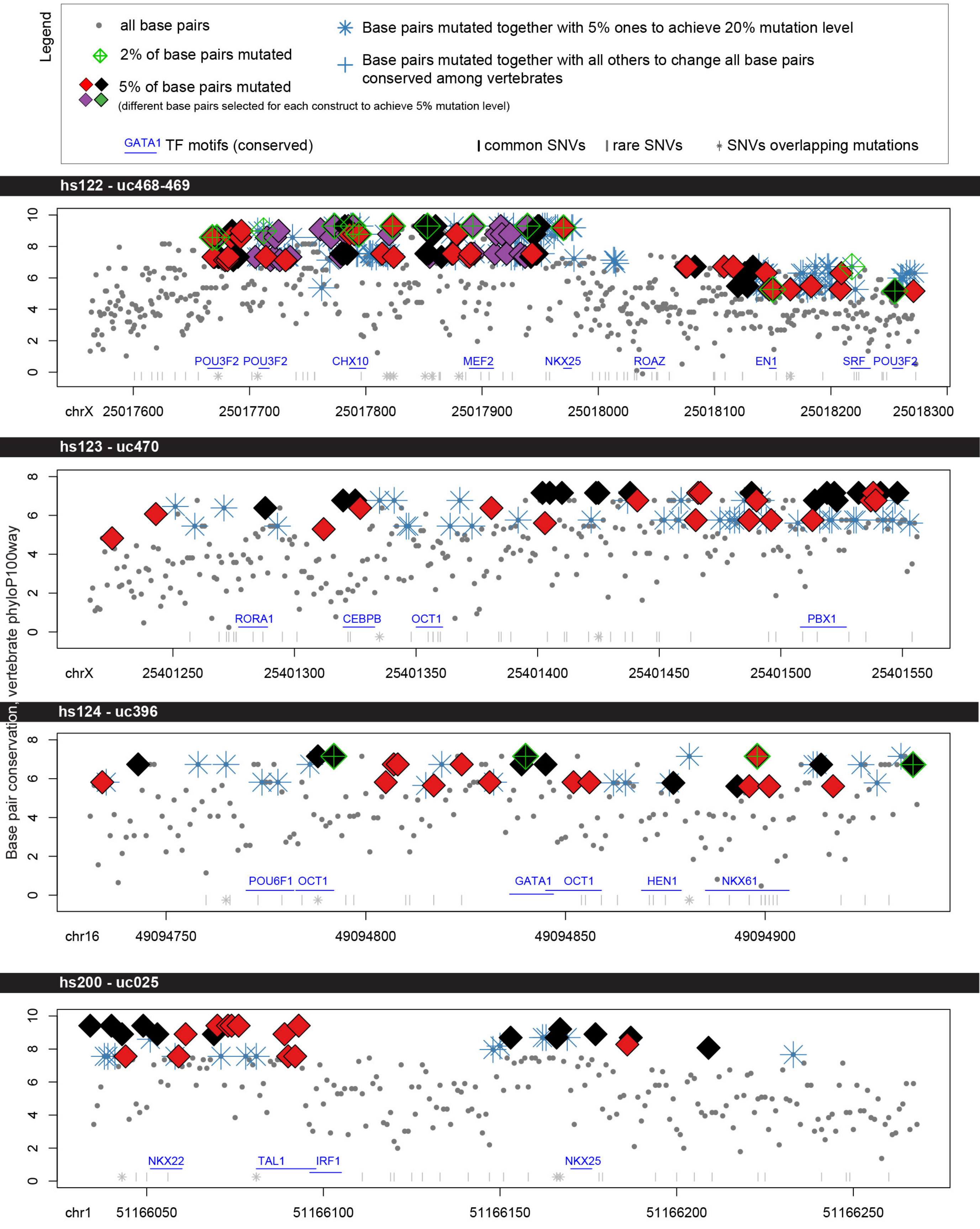
Supplementary Figure 1 | Snetkova et al.



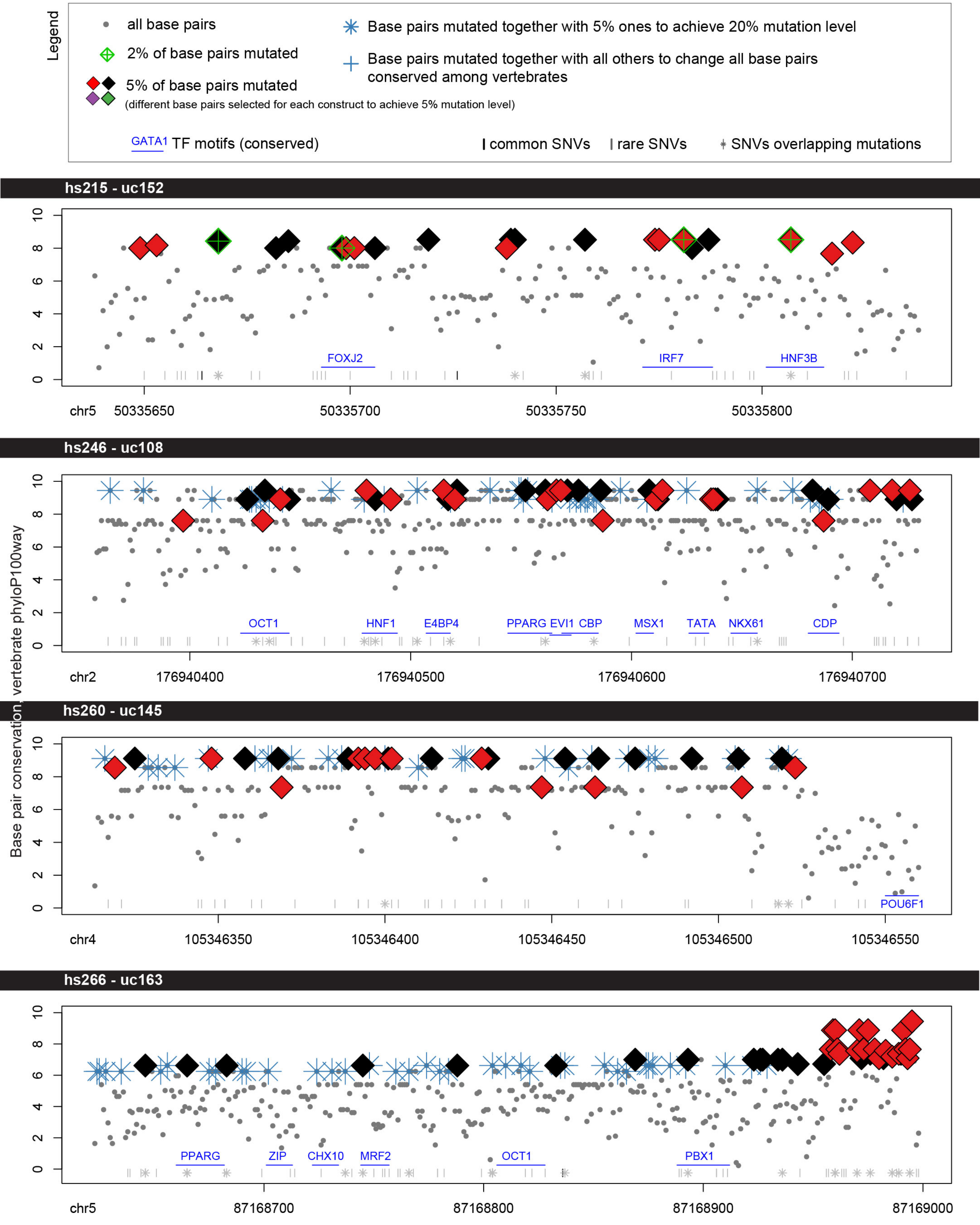
Supplementary Figure 2 | Characterization of base pairs selected for mutagenesis for 23 ultraconserved enhancers.



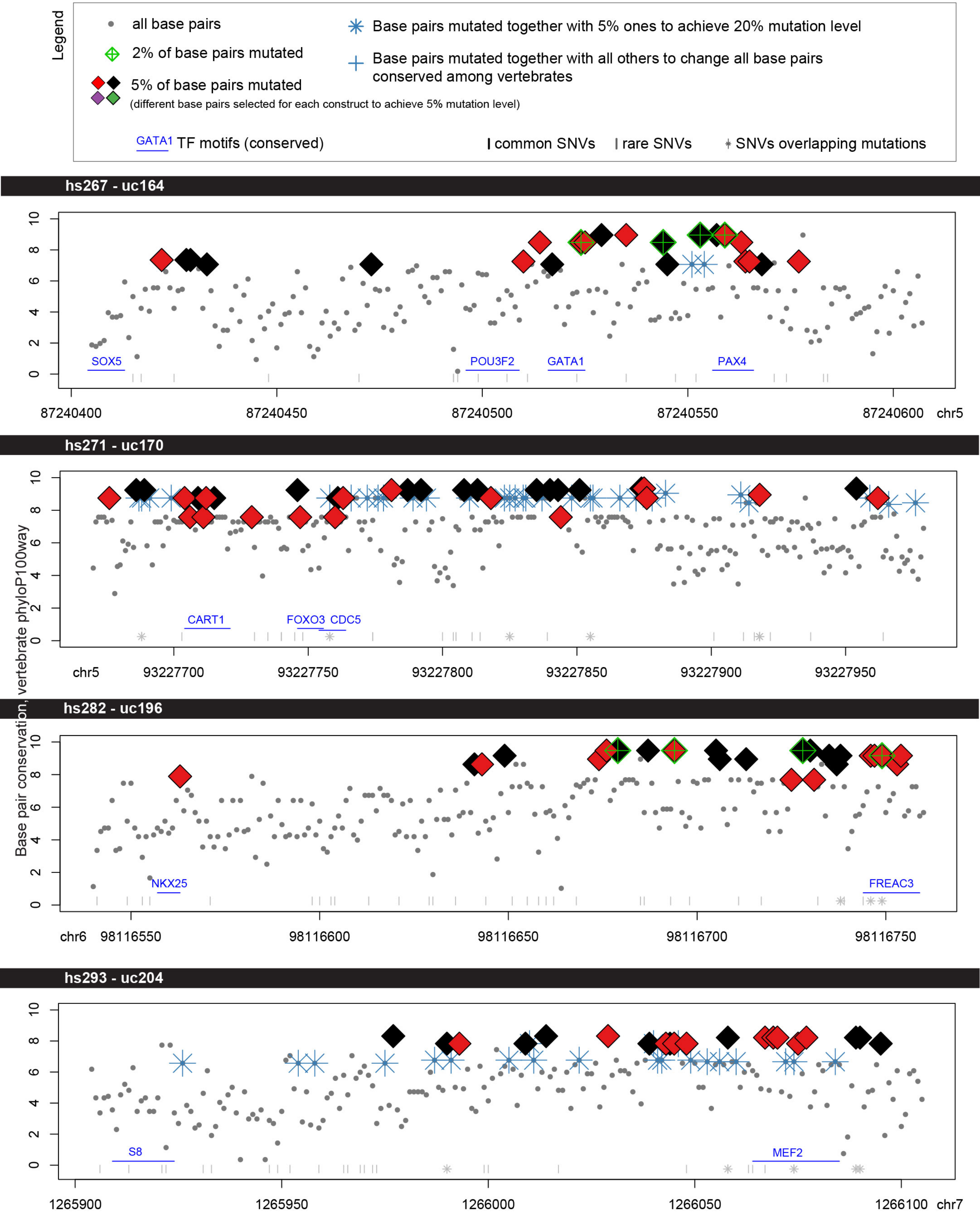
Supplementary Figure 2 | Snetkova et al.



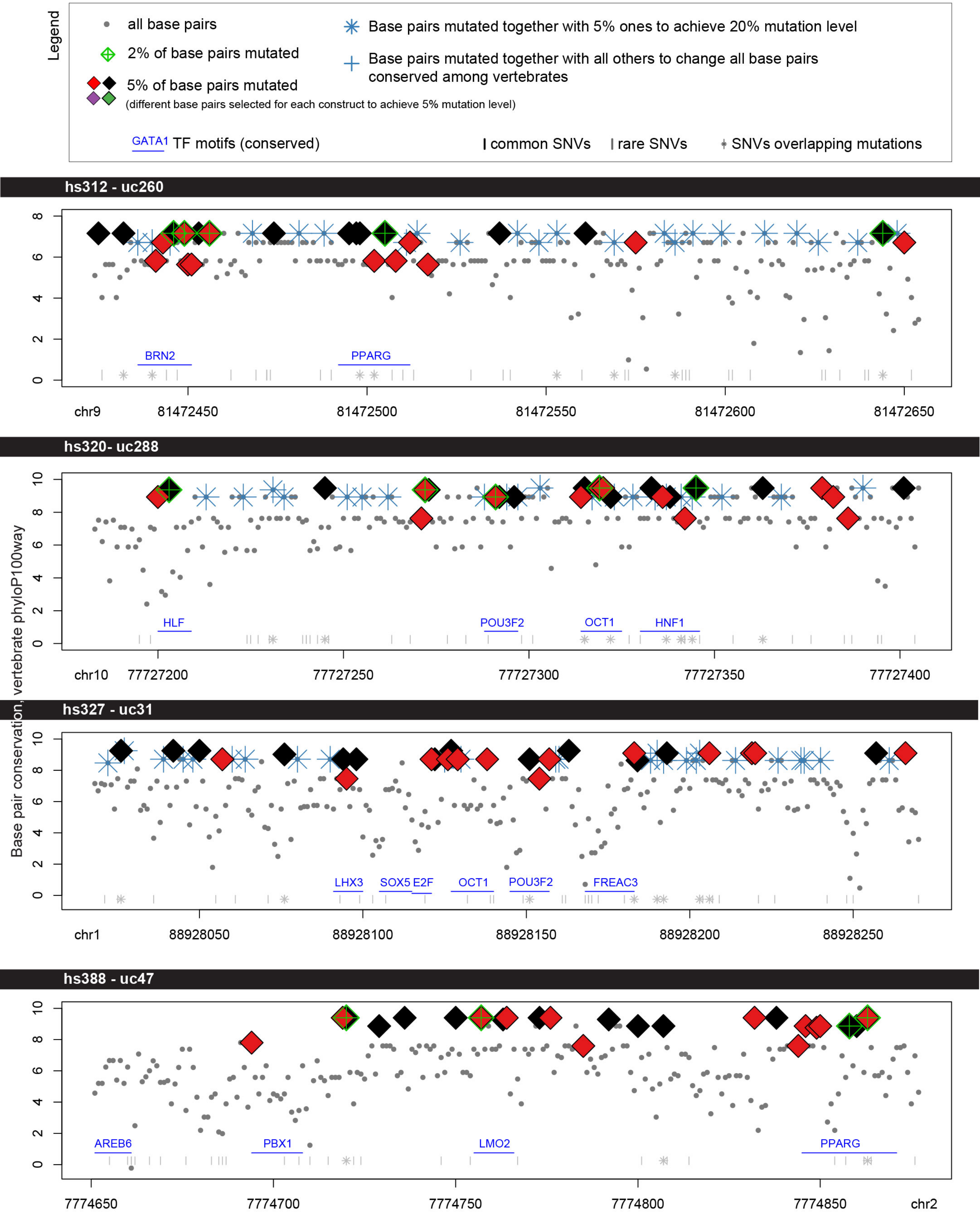
Supplementary Figure 2 | Snetkova et al.



Supplementary Figure 2 | Snetkova et al.



Supplementary Figure 2 | Snetkova et al.



Supplementary Figure 2 | Snetkova et al.