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Supplemental Information

Homeodomain Protein Otp and Activity-Dependent Splicing Modulate Neuronal Adaptation to Stress

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Supplemental Inventory

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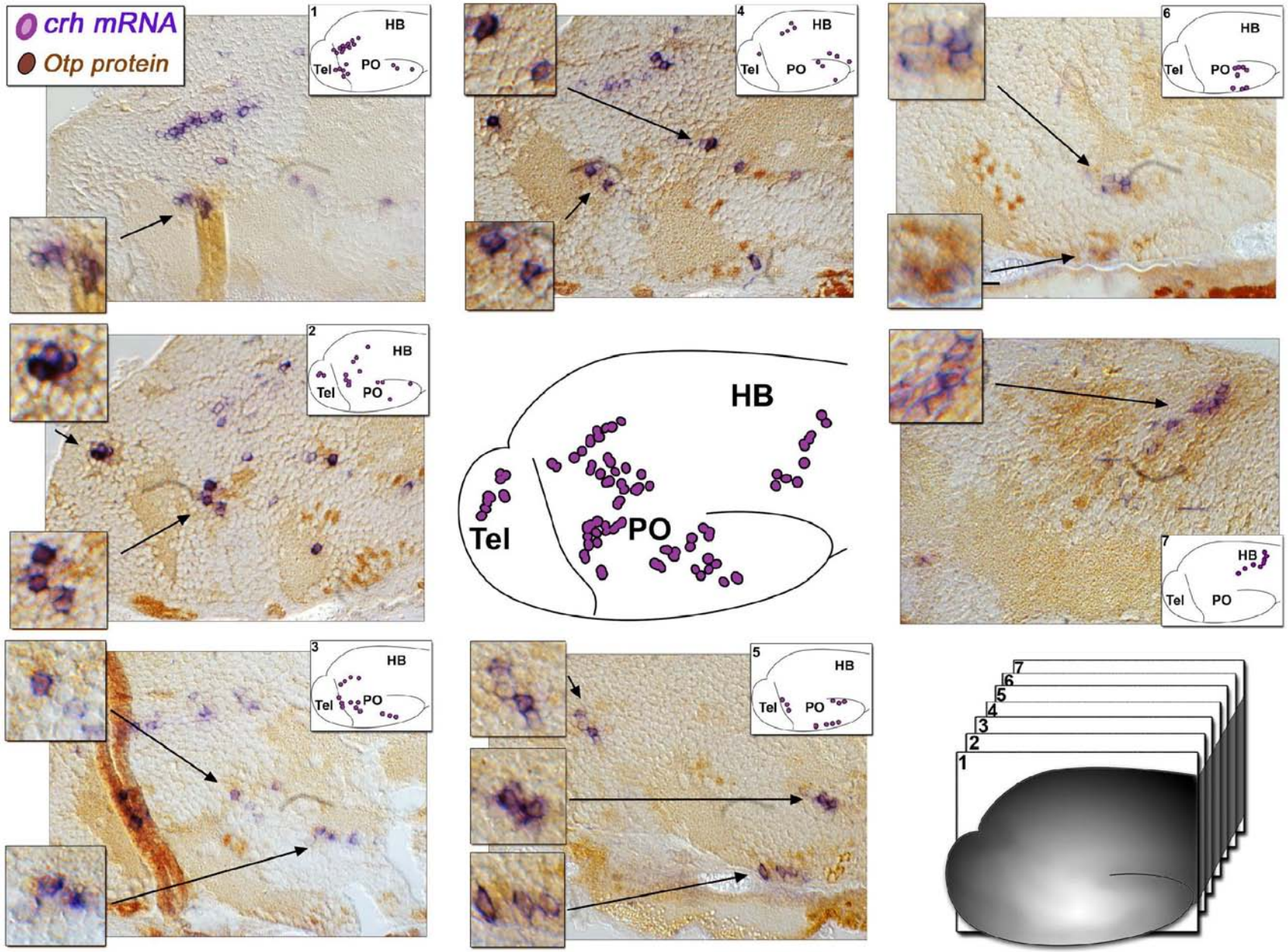
crh mRNA
Otp protein
HB
Tel
PO
1
2
3
4
5
6
7

Figure S1. Expression of Otp and CRH in 6-day-old Zebrafish Larva, Related to Figure 1

Serial sagittal sections (6 μm) of 6-day old zebrafish larva subjected to whole mount *in situ* hybridization with a *crh*- directed probe (purple) followed by paraffin embedment and sectioning, antigen retrival and immuno-staining with an anti-Otp antibody (brown). The scheme in the center of the figure depicts the location of the major CRH+ neuronal clusters in 6-day old larva, whereas schemes shown in the insets of the image panels depict the subsets of CRH+ cells in each of the tissue sections. High-magnifications of *crh*+;Otp+ neuronal clusters (arrows) are also presented.

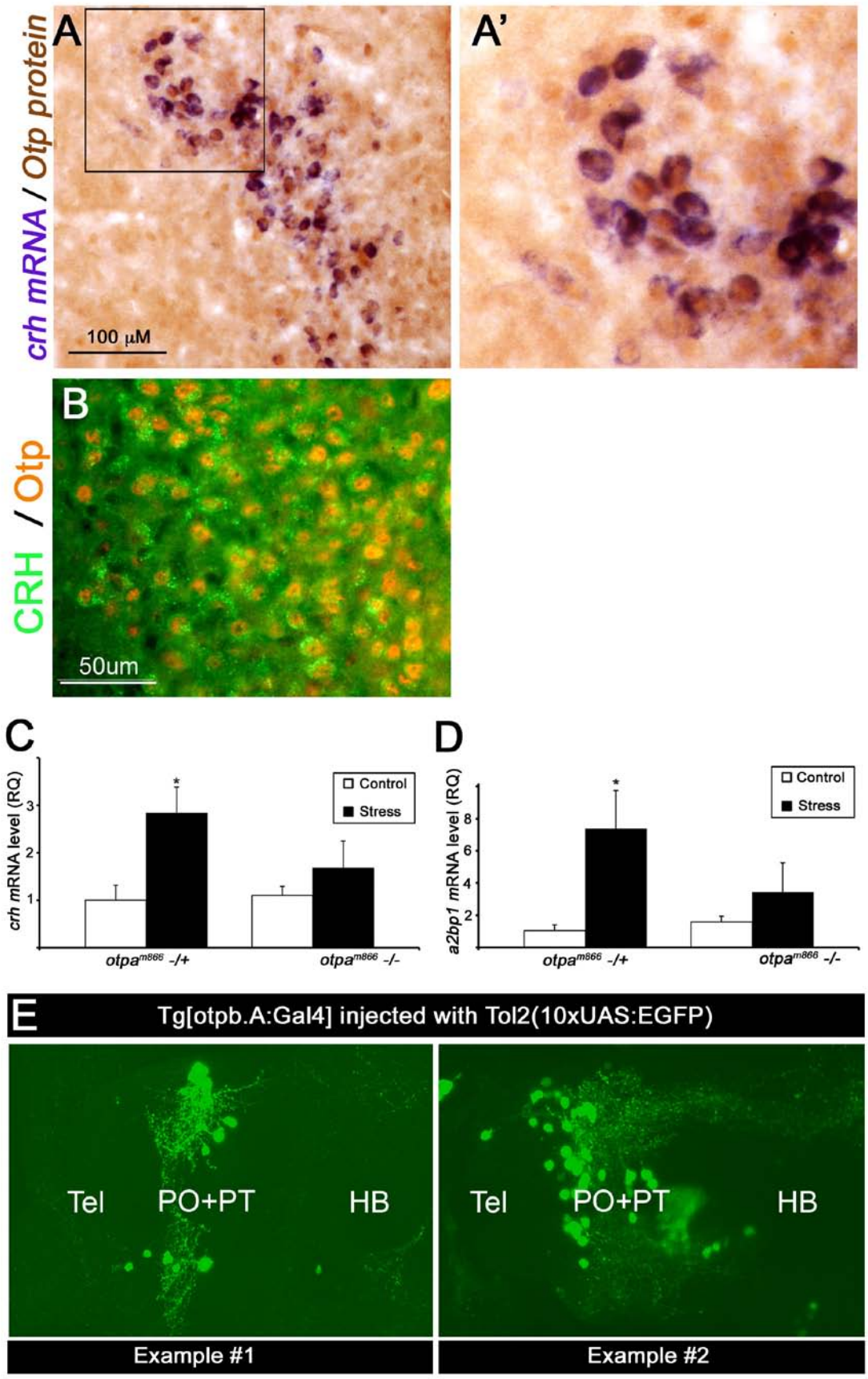
A
A'
crh mRNA / Otp protein
100 μM
B
CRH / Otp
50um
C
crh mRNA level (RQ)
Control
Stress
otpa^m866 -/+
otpa^m866 -/-
D
a2bp1 mRNA level (RQ)
Control
Stress
otpa^m866 -/+
otpa^m866 -/-
E
Tg[otpb.A:Gal4] injected with Tol2(10xUAS:EGFP)
Tel
PO+PT
HB
Example #1
Tel
PO+PT
HB
Example #2

Figure S2. Expression of Otp and CRH in the Mouse PVN and Levels of Zebrafish crh in Response to Osmotic Challenge, Related to Figure 1

(A and B) Expression of Otp and CRH in the mouse PVN. Coronal sections through the adult mouse hypothalamus showing colocalization of Otp and *crh* in the mouse paraventricular nucleus.

(A) Floating brain sections were subjected to *in situ* hybridization with a *crh*- directed probe followed by immuno-staining with an anti-Otp antibody. High-magnification of the *crh*+;Otp+ area (black rectangle) is shown in panel A'.

(B) Double immuno-fluorescence staining with guinea pig anti-CRH antibogy (Bachem California, Torrance, USA) together with rabbit anti-Otp antibody, which was generated in our lab.

(C and D) Attenuated osmotic stress response in $otpa^{m866}$ mutant. Osmotic stress challenge was applied to 6 day-old progenies of a $otpa^{m866}$-/+ cross by incubation in 50% artificial seawater for 4 min (See details in the *Supporting Material*). The amounts of *crh* (C, n=6) and *a2bp1* (D, n=5) mRNAs were measured in control and stressed fish larvae using quantitative PCR (qPCR). Larvae were genotyped by sequencing and *crh* mRNA levels of mutant (-/-) and heterozygous (-/+) animals were plotted accordingly. *$p<0.05$.

(E) Representative examples of *otp*:Gal4 transgenic line that was co-injected with transposon-based (Tol2) vector harboring EGFP under the control of UAS elements together with *transposase* mRNA. EGFP expression is readily visible in 6-day old larvae.

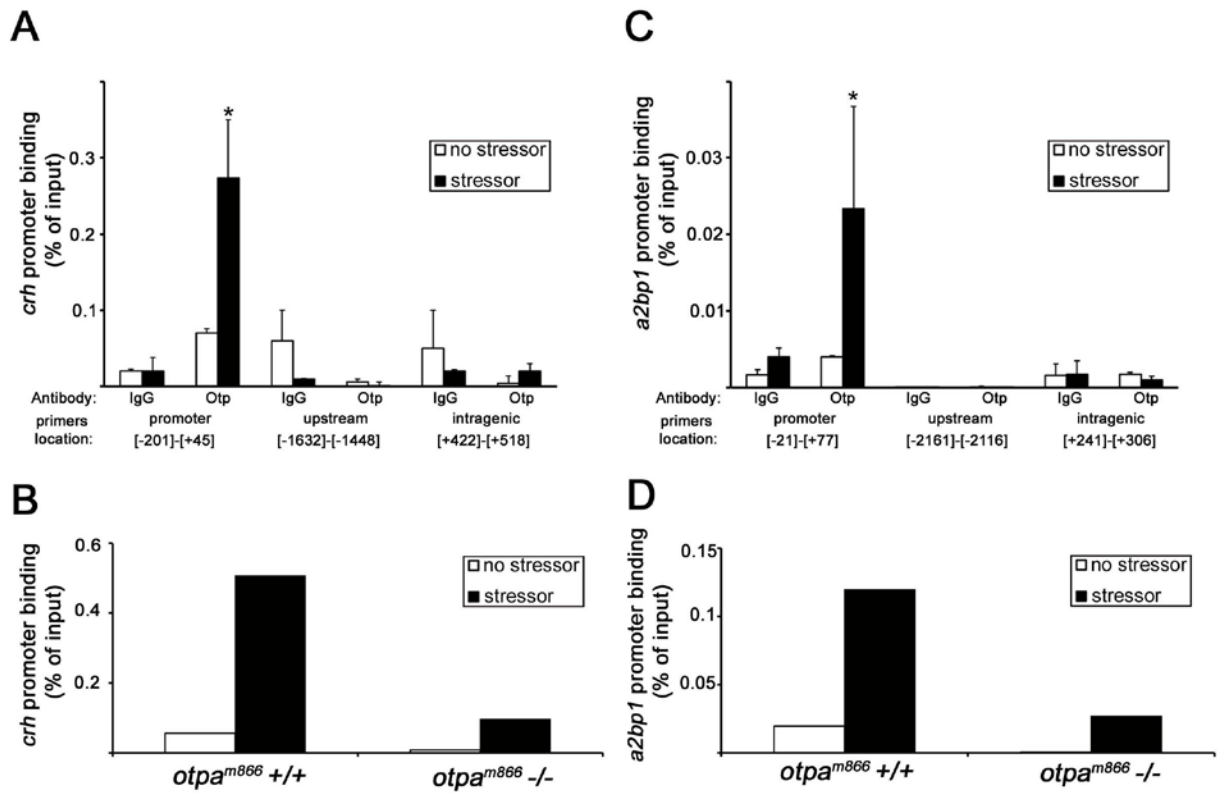

Figure S3. Specificity of Otp Association with *crh* and *a2bp1/rbfox1* in Zebrafish Larvae, Related to Figure 3

(A and C) Histograms showing quantitative ChIP analyses of the recruitment of Otp to *crh* (A, **p*<0.05, n=3) and *a2bp1* (C, **p*≤0.1, n=3) promoters compared to remote upstream and intragenic regions within the *crh* and *a2bp1* gene loci and compared to binding of control rabbit IgG antibody. Chromatin was extracted from a pool of 50 larvae per treatment 30 min after physical challenges, followed by anti-Otp ChIP.

(B and D) ChIP analysis of Otp in the *otpa*^{m866} mutant allele. Histograms showing quantitative ChIP analyses of the recruitment Otp to *crh* (B) and *a2bp1* (D) promoters. Chromatin was extracted from a pool of 50 larvae per treatment 30 min after physical challenges, followed by anti-Otp ChIP. The genomic locations [relative to the transcription start (+1) site] of the oligonucleotide primers used for ChIP analyses are shown at the bottom and their sequences are presented in the Table S2.

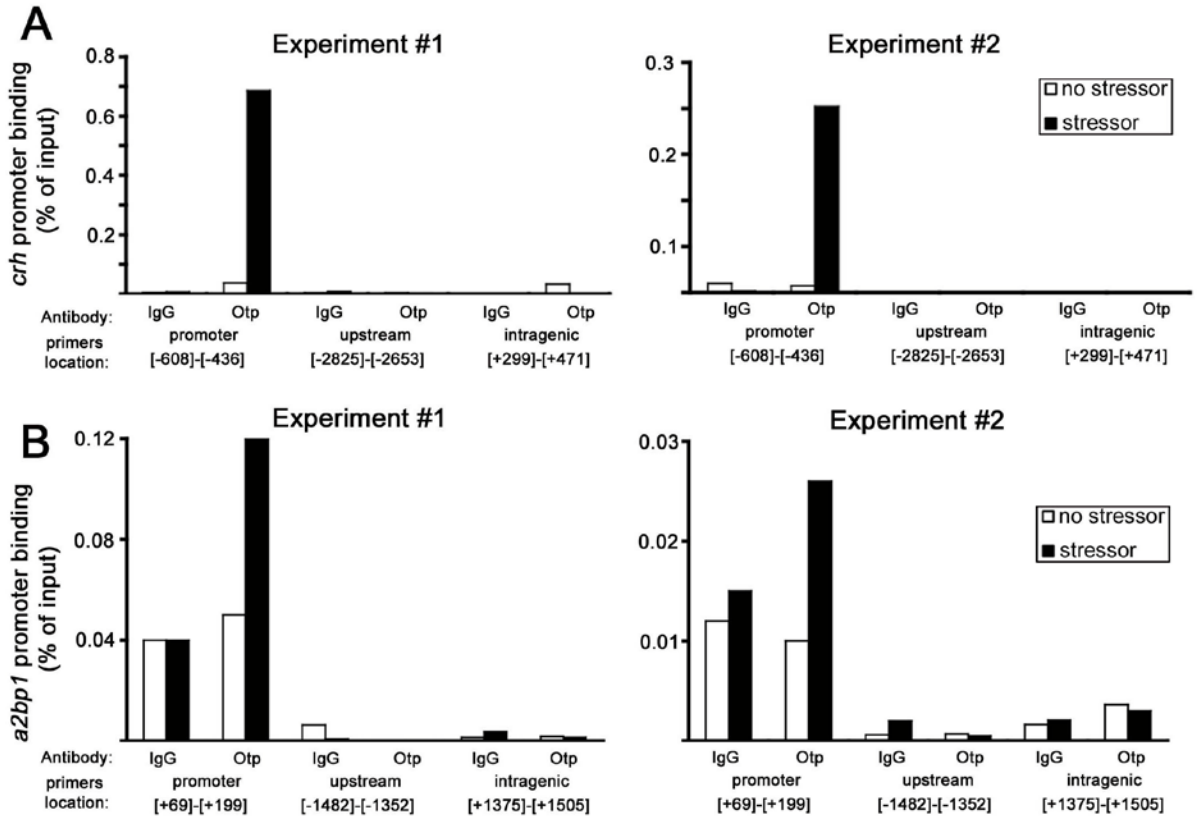

Figure S4. Otp Is Recruited to the Mouse *crh* and *a2bp1/rbfox1* Promoters following Psychological Stressors, Related to Figure 3

Histograms showing quantitative ChIP analyses of the recruitment of Otp to *crh* (A) or *a2bp1* (B) gene loci in two independent experiments. Mice were subjected to restraint stress or left unchallenged and PVN were dissected from mice 30 and 60 min after the initiation of the respective restraint (A) and foot shock (B) stressors. Chromatin was extracted from a pool of 5 animals per treatment followed by anti-Otp ChIP. Recruitment of Otp to the respective promoter was assessed by quantitative PCR and calculated relative to the amount of input chromatin.

The specificity of Otp association with *crh* and *a2bp1* promoters is demonstrated by the lack of Otp binding to remote upstream and intragenic regions within the *crh* and *a2bp1* gene loci and by comparing to binding of control rabbit IgG antibody. The genomic locations [relative to the transcription start (+1) site] of the oligonucleotide primers used for ChIP analyses are shown at the bottom and their sequences are presented in the Supplemental Table S2.

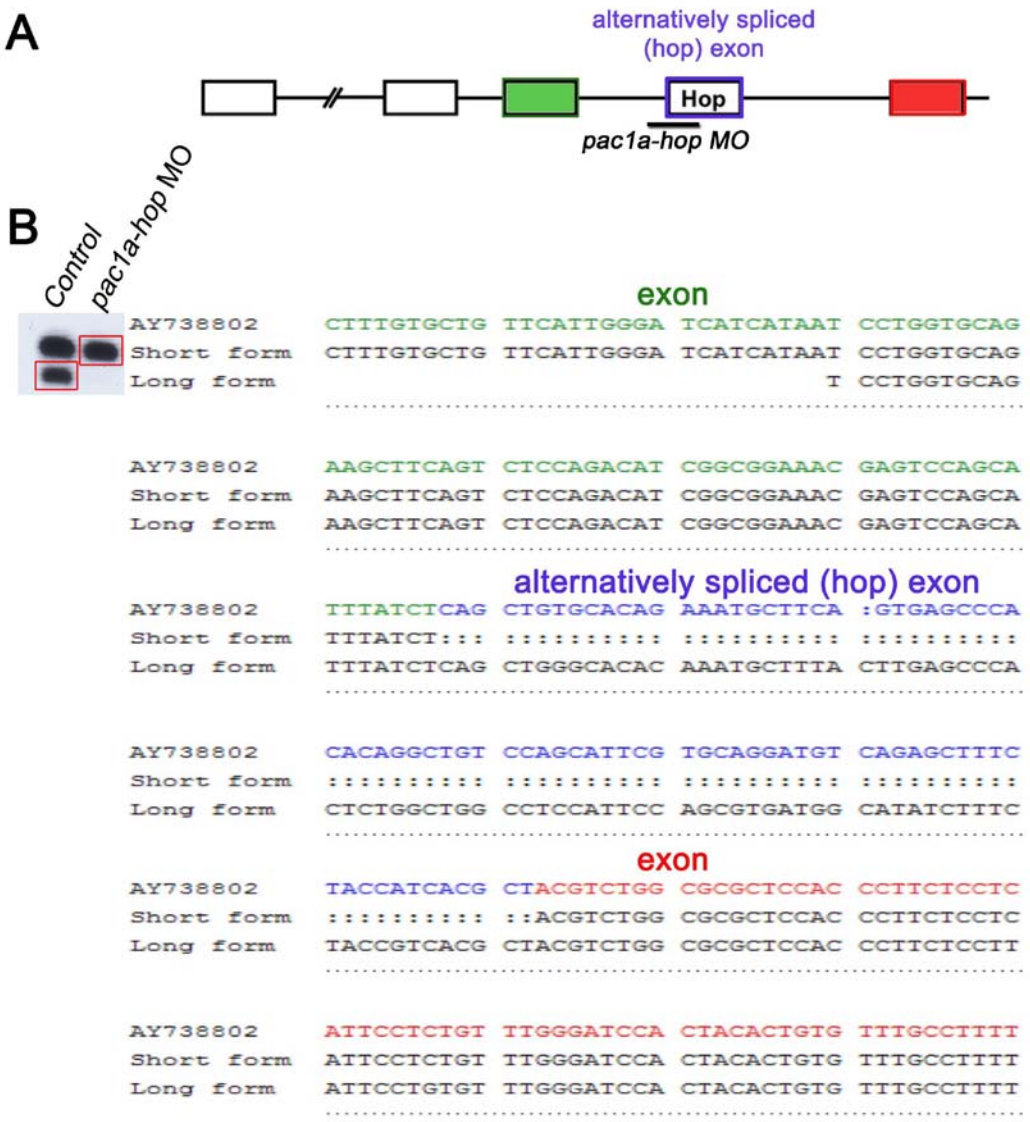

Figure S5. Inhibition of Alternative Splicing by *pac1a-hop* Antisense Morpholinos, Related to Figure 6

(A) Scheme depicting *pac1a* gene structure as well as antisense MO binding site. Alternative splicing of the zebrafish *pac1a* gene was blocked by microinjecting (at 1.5 ng/1.7 nl) synthetic antisense morpholino oligonucleotides (*pac1a-hop* MO, Gene Tools, LLC, Corvallis, OR).

(B) *pac1a* cDNA fragments were amplified from either control or larvae (6 day-old), which were injected with *pac1a-hop* MO followed by gel electrophoresis. The image on the left shows the short (*pac1a*) and long (*pac1a-hop*) splice variant in the control sample, while only the short isoform, which lacks the exon encoding to the '*hop*' cassette is detected in *pac1a-hop* MO- injected sample. DNA fragments of the short and long isoforms, marked by red rectangles, were excised from the gel and analyzed by sequencing to confirm the integrity of *pac1a* reading frame in *pac1a-hop* MO- injected larvae. The oligonucleotide primers used to amplify the cDNA fragments of *pac1a* splice variants were had the following sequences: Forward: ATGAATGATAACACTGCCCTC, Reverse: CCAGGCCGAGCTCAAAGACC.

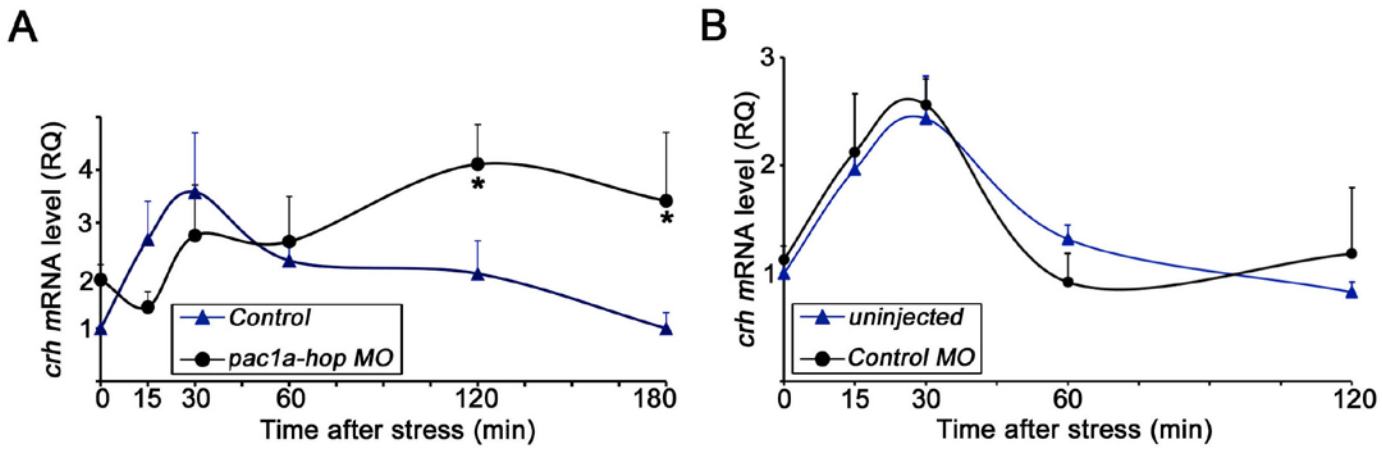

Figure S6. Effects of *pac1a-hop* and Control Morpholino Oligonucleotides on the Kinetics of Stress-Induced *crh* Levels, Related to Figure 6

Osmotic (A) or physical (B) stress challenges (see '*Experimental Procedures'*) were applied to mock-treated larvae (A, '*Control'*) or uninjected larvae (B) or their respective siblings, which were injected (each embryo at 1.5 ng/1.7 nl) with either splice-blocking *pac1a-hop* MO (A) or unrelated morpholino oligonucleotide (B).

The amount of *crh* mRNA was measured in individuals fish larva (A, n=9) or in pools of 10 larva per treatment (B, n=3) different time points of recovery using quantitative PCR (qPCR). *$p \leq 0.05$

Supplemental Experimental Procedures

Table S1. Oligonucleotide Primers for Quantitative PCR Analysis

Zebrafish

Gene	Primers	Acession Number	Fragment size (bp)
crh	ccgatttccctagatctgac cactatggtacagagtattc	NM_001007379	190
a2bp1/rbfox-1	catatttaatgaacgagg ccgtgccgtgtaatttctc	NM_001005596	95
pac1a-hop (long)	gagctttctaccatcacgct cccgcttgctgaaatcctc	AY738800	112
β-actin	gaggctctcttccagccttc cggatgtccacgtcgcacttc	NM_131031	95

Mouse

Gene	Primers	Acession Number	Fragment size (bp)
crh	gcatgcacaaagtgtatttc ctttaagatatcgctataaag	NT_162143	105
a2bp1/rbfox1	ctgcgcttcagaccaggtg gttggcttctctcactccag	NM_021477	130
pac1-hop (long)	gcaatgagtcgagcatctac gagtaatggtggatagttctg	NM_007407	105
pac1 (short)	ggccagccgtaagtagatgctc ctataatggttaactttgtg	NM_001025372	112
hprt	gcagtacagccccaaaatgg ggtccttttcaccagcaagct	J00423	52

Table S2. Oligonucleotide Primers for Quantitative ChIP Analysis

Zebrafish

Gene	Primers	ZFIN ID	Fragment size (bp)
crh	gtgtggattcaatctgaagg gtttgacacctcttgtctaag	ZDB-GENE-041114-75	246
crh - upstream	gcatgacatgctgcacattc ggccaagtaaatccacttc	ZDB-GENE-041114-75	184
crh - intragenic	ctgttggaggggaaagttgg ctcctccgacctgcgctc	ZDB-GENE-041114-75	96
a2bp1/rbfox-1	actcgctacacacacagc ctaagcagctgccttagtc	ZDB-GENE-040927-11	98
a2bp1/rbfox-1 upstream	gttccaaacctttctctgtac gaagaaagctgaaactgtta	ZDB-GENE-040927-11	45
a2bp1/rbfox-1 intragenic	catttactacggattaagtc gcaacaaaccatttaagttc	ZDB-GENE-040927-11	65

Mouse

Gene	Primers	Gene ID	Fragment size (bp)
crh	cctaaagaacccagttcagg gttcctcccacctctaaaac	12918	172
crh - upstream	ggagcaacacacatatcagc ctaacatgaaaatagcagtg	12918	140
crh - intragenic	gcaaggcaggcaggacgac gagcgcccctaacatgcggc	12918	79
a2bp1/rbfox-1	ctcaggacaaatgccttctc caacatgactaaccaaatac	268859	99
a2bp1/rbfox-1 upstream	caccatgattccacgccatg ccgaaatgaaaggcattgag	268859	120
a2bp1/rbfox-1 intragenic	ggatactattgctacacttc caagttcccttccttggtgc	268859	78

Table S3. Antisense Morpholino Knockdown Oligonucleotides

Name	Location	Sequence
pac1a-ATG	translation (ATG) start	TCGCCGCTTGTCTATACATTCTGCT
pac1a-hop	hop splice site	AGCTGCTAAACACGACAAAGACAAC
Control MO	no match in genome	ATGACACTGGACCCCACTCACCTCC