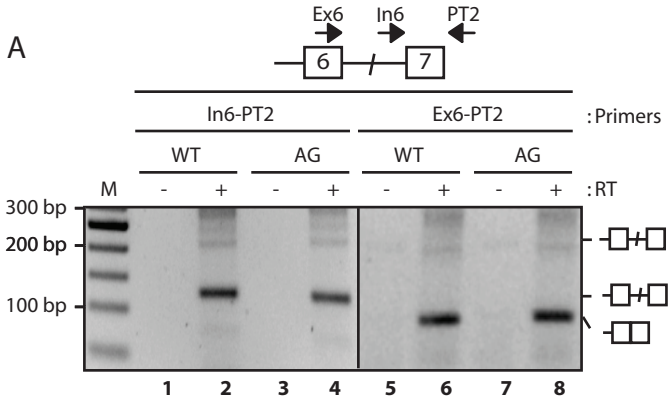
Corrionero et al

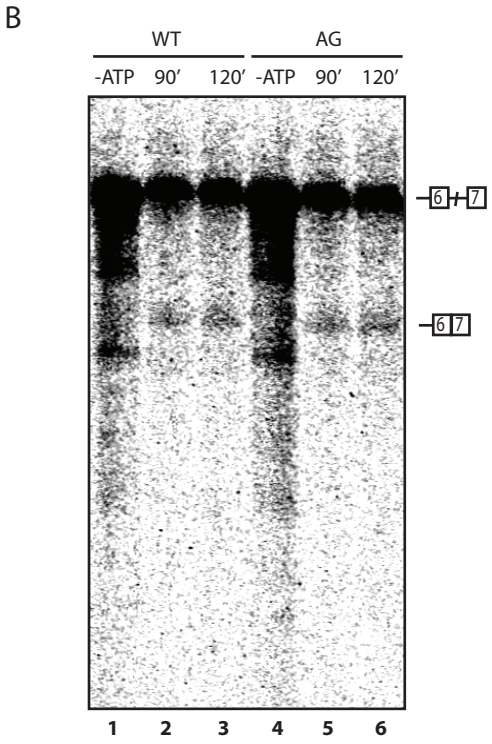
Supplementary Figure Legends

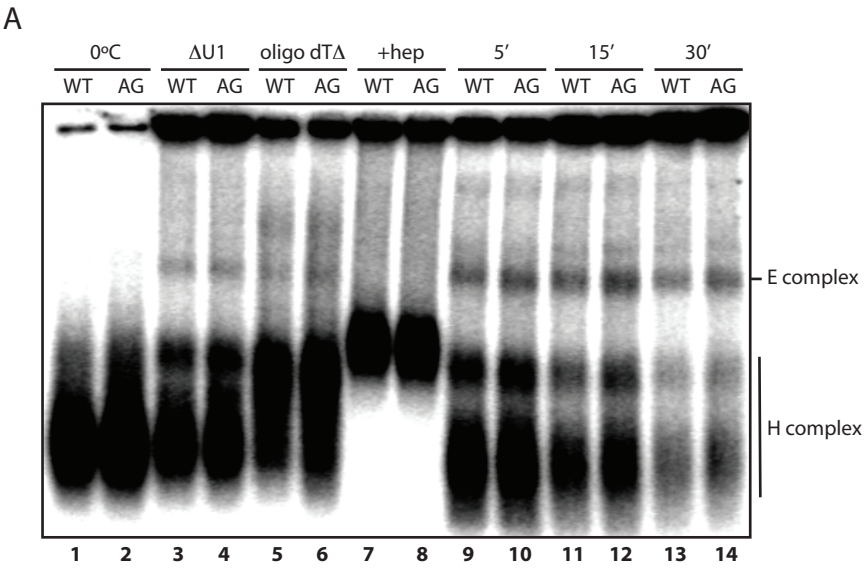
Supplementary Figure 1. The AG mutation does not affect intron 6 splicing. (**A**) Splicing pattern of wild type (WT) and AG intron 6-containing minigenes. RNA from HeLa cells transfected with the minigenes utilized in Fig. 1B (lanes 3 and 4) was analyzed by semi-quantative PCR using primers specific to detect either intron-containing transcripts (T7-In6: GCTAATACGACTCACTATAGGGCTGAGACCTGAGTTGATA; and PT2 (corresponding to vector sequences: AAGCTTGCATCGAATCAGTAG) or the spliced product (Ex6: TTTGCCAATTCCACTAATTGTTTG; PT2: AAGCTTGCATCGAATCAGTAG). The position of these primers is indicated by arrows at the top. The cDNA was amplified for 30 cycles (for detection of unspliced transcripts) and 25 cycles (for detection of spliced transcripts). The positions of size markers and amplification products are indicated. We conclude that the AG mutation does not compromise splicing of intron 6. (**B**) In vitro pre-mRNA splicing of intron 6-containing wild type and AG transcripts . Uniformly α^{32}P-UTP labeled RNAs corresponding to wild type (WT) or AG mutant Fas genomic region between exons 6 and 7, including the 3' 68 nucleotides of intron 5 and a deletion of intron 6 (50-1102) (Izquierdo et al., 2005), were incubated with HeLa cell nuclear extracts under splicing conditions (see Materials and Methods) in the presence of 4 mM MgCl2 and in the absence or presence of ATP for 90 or 120 minutes. RNA species were purified and analyzed by electrophoresis in 13% denaturing polyacrylamide gels. The positions of pre-mRNA and spliced product are indicated. We conclude that the AG mutation does not affect splicing of intron 6 in vitro.

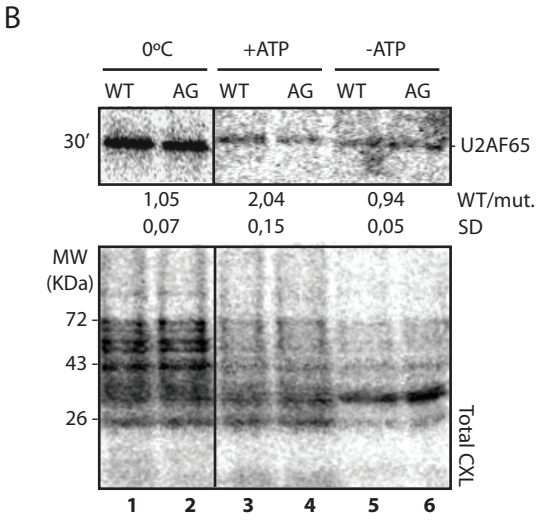
Supplementary Figure 2. (**A**) **The AG mutation does not compromise E complex formation.** Uniformly α-^{32}P-UTP labeled WT or AG RNAs corresponding to exon 6, the 3' 68 nucleotides of intron 5 and the 5' 25 nucleotides of intron 6 were incubated under splicing conditions in the absence of ATP for 5, 15 or 30 minutes and E complex assembly was analyzed by electrophoresis in 1.5% low melting point agarose gels as described in Das and Reed, RNA 5: 1504-1508 (1999). Controls for E complex formation included incubation at 0ºC (lanes 1 and 2), the use of nuclear extracts were U1 snRNA was inactivated by RNase H treatment (lanes 3 and 4), the use of U2AF-depleted nuclear extracts (oligo dTΔ, lanes 5 and 6) [Guth et al., MCB 19: 8263-71 (1999)] or treatement with 5 μg/μl heparin for 10 min at room temperature after incubation of the reaction at

30ºC (lanes 7 and 8). Under all these circumstances the complex detected was severely impaired indicating that formation of the complex requires U1 snRNP, U2AF, incubation at 30ºC and is heparin-sensitive, all hallmarks of E complex formation. We conclude that the AG mutation does not compromise E complex formation. (**B**) Destabilization of U2AF65 crooslinking by the AG mutation is ATP- and temperature-dependent. U2AF65 crosslinking-immunoprecipitation to wild type and mutant AG was carried out as in Figure 3A using α^{32}P-UTP labeled wild type and AG RNAs comprising the 3′ 68 nucleotides of intron 5, exon 6, and 5' 25 nucleotides of intron 6, incubated with nuclear extracts at 0ºC or under splicing conditions in the presence or absence of ATP at 30ºC for 30'. UV-mediated crosslinking, immunoprecipitation of U2AF65 and analysis of the crosslinked species after 15 minutes of incubation was carried out as described in Fig. 3A. The position of RNA-crosslinked U2AF65 is indicated. An aliquot of the total crosslinking befote immunoprecipitation was used as loading control. While reduced U2AF65 crosslinking is observed at 15 minutes in the AG mutant compared to control under standard conditions (consistent with the results of Figure 3), such differences are not observed at 0ºC or in the absence of ATP. We conclude that the reduction in the interaction of U2AF65 with the AG mutant pre-mRNA after 15 minutes of incubation is temperature and ATP-dependent.


A
Ex6
In6
PT2
6
7
In6-PT2
Ex6-PT2
: Primers
WT
AG
WT
AG
M
- + - + - + - + : RT
300 bp
200 bp
100 bp
1 2 3 4 5 6 7 8


B
WT
AG
-ATP 90′ 120′ -ATP 90′ 120′
6 7
6 7
1 2 3 4 5 6


A
0ºC
ΔU1
oligo dTΔ
+hep
5′
15′
30′
WT AG WT AG WT AG WT AG WT AG WT AG WT AG
E complex
H complex
1 2 3 4 5 6 7 8 9 10 11 12 13 14


B
0ºC
+ATP
-ATP
WT AG WT AG WT AG
30′
U2AF65
1,05 2,04 0,94 WT/mut.
0,07 0,15 0,05 SD
MW (KDa)
72
43
26
Total CXL
1 2 3 4 5 6