Supplemental material JCB

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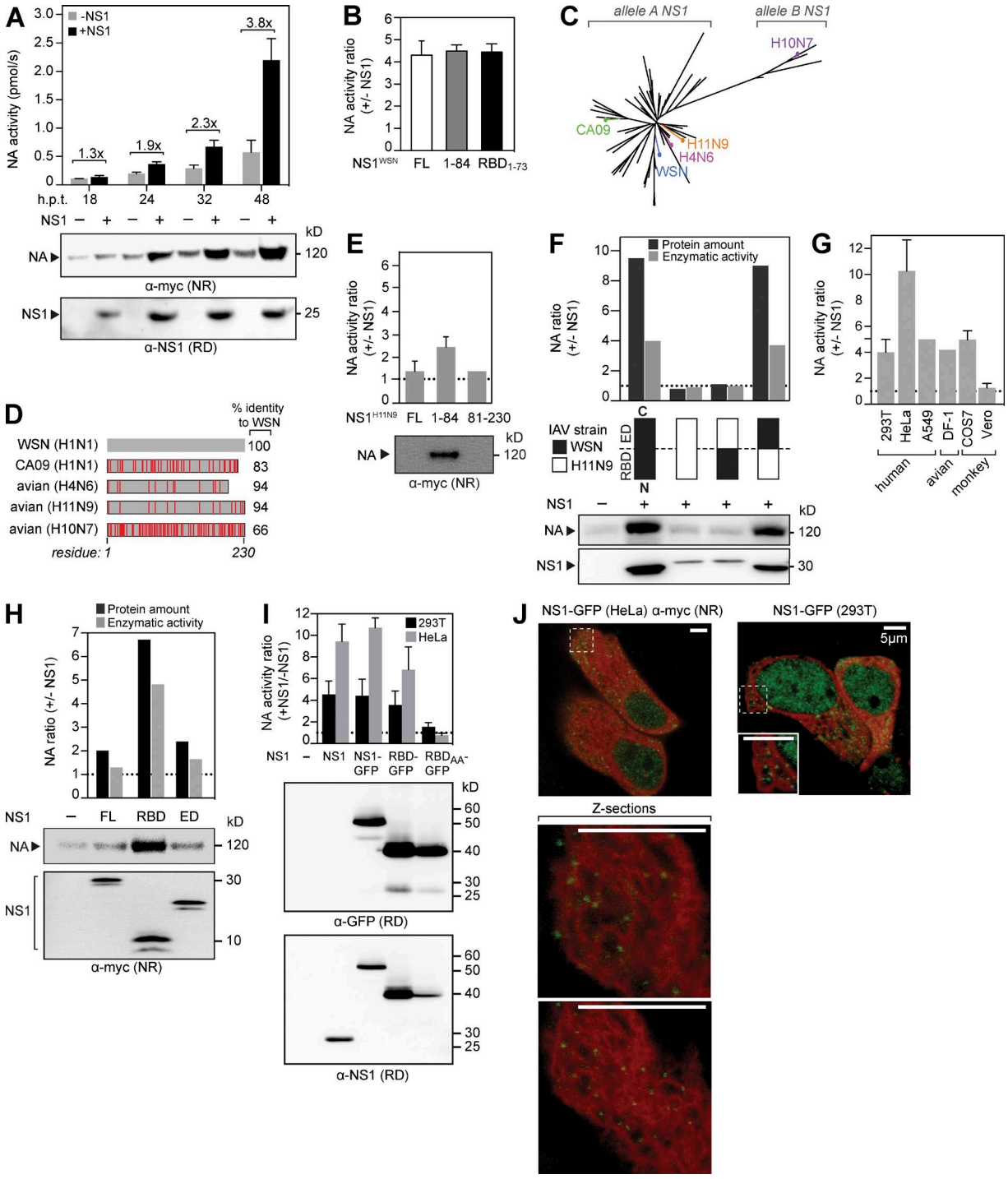

Figure S1. **The properties of NS1 that affect NA regulation.** (A) NA^{WSN} was transfected alone or with $NS1^{WSN}$ in 293T cells. Cells were harvested at the indicated times after transfection (h post transfection; h.p.t.), and NA protein (immunoblot) and activity levels were measured (bar graph). (B) NA^{WSN} was transfected alone or with full-length (FL) NS1, and the RBD with the linker region (amino acids 1–84), or the RBD (amino acids 1–73), and the change in NA activity levels was determined 48 h after transfection. (C) NS1 protein sequences from avian and human IAVs were retrieved from the NCBI Influenza Virus Resource, clustered with an identity of 0.95 with usearch, and aligned with mafft. The NS1 allele A and B clusters are shown with the sequences used in this study. (D) Illustration of the amino acid variation (red lines) between the studied NS1 constructs and $NS1^{WSN}$. Percent identity was calculated compared with $NS1^{WSN}$ (excluding deletions). (E) NA^{WSN} was transfected alone or with indicated $NS1^{H11N9}$ constructs in 293T cells. NA activity changes are shown with an NA immunoblot. (F) NA^{WSN} was cotransfected with the indicated RBD and ED chimeras of $NS1^{WSN}$ and $NS1^{H11N9}$. NA protein and activity levels were normalized to cells expressing NA. (G) NA^{WSN} was transfected alone or with $NS1^{WSN}$ in the indicated cell lines, and at 48 h after transfection, the changes in NA activity levels were determined with respect to NS1 coexpression. (H) NA^{WSN} was cotransfected in Vero cells with $NS1^{WSN}$, the RBD, or ED, and the NA protein and activity levels were analyzed. (I) NA was cotransfected with C-terminal GFP-tagged $NS1^{WSN}$, the RBD, or RBD_{AA} in 293T and HeLa cells. Cells were harvested 48 h after transfection, then NA activity was measured, and synthesis was confirmed by immunoblotting with GFP and NS1 antisera. (J) Live images of HeLa (left) and 293T (right) cells were taken 48 h after transfection with NS1-GFP and the ER membrane marker mCherry-Sec61β. Imaging was performed with an LSM700 inverted microscope and a 63× objective. Enlarged sections (boxed regions in main images) of the indicated ER membrane regions are shown as z sections (HeLa) or an inset (293T). Error bars indicate SD. *n* = 3. NR, nonreduced; RD, reduced.

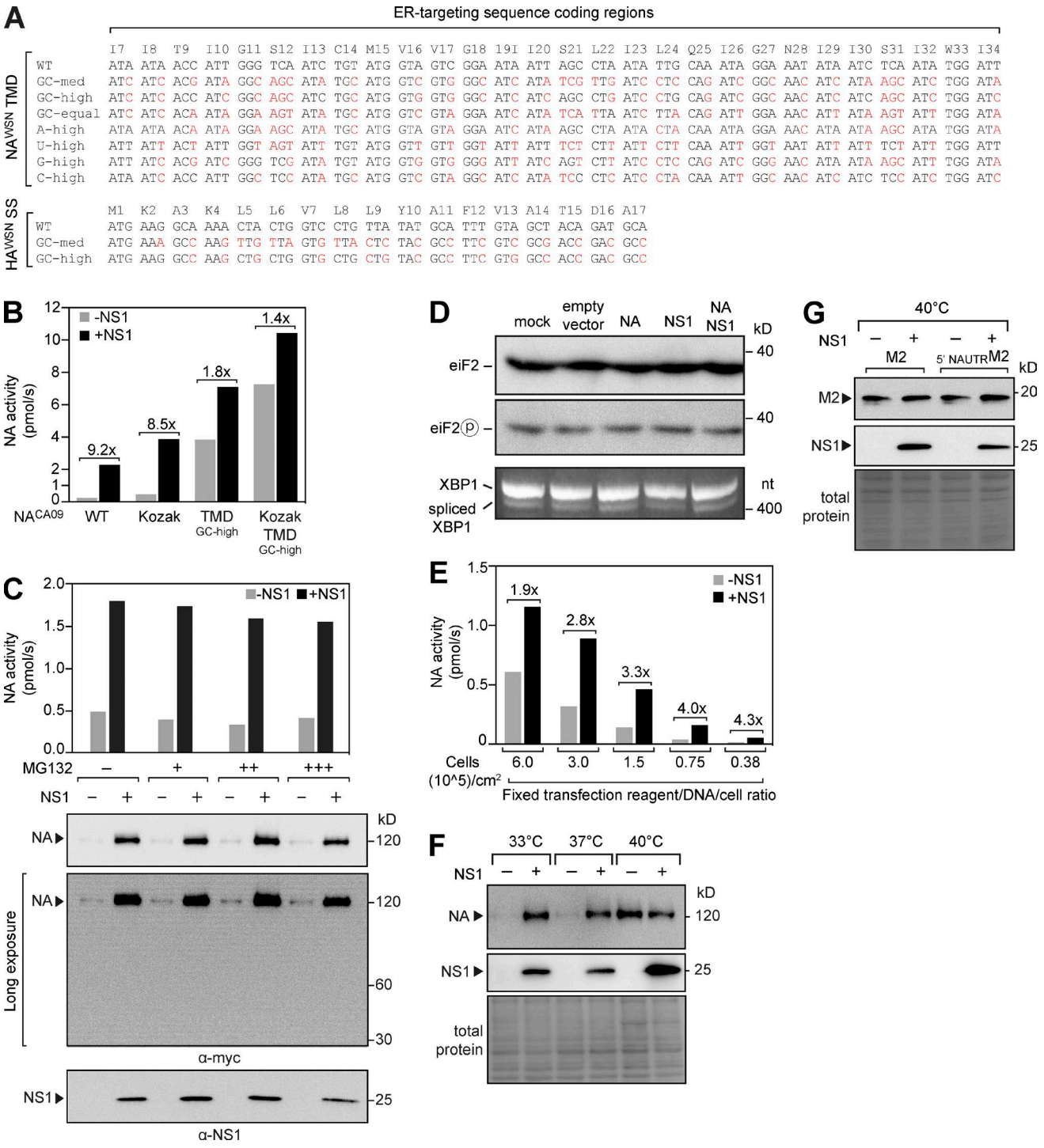

Figure S2. **NA regulation is dependent on the ER-targeting sequence coding regions, cell growth, and temperature.** (A) The synonymous mutations (red) that were introduced into the ER-targeting sequence coding regions of the indicated NA and HA constructs are displayed. (B) The NA^{CA09} constructs were transfected alone or with $NS1^{WSN}$ in 293T cells, and NA activity was measured. The stronger Kozak ribosomal binding sequence (GCCACC) was inserted before the NA start codon. NA^{CA09} $TMD_{GC\text{-}high}$ was designed by increasing GC content using synonymous mutations in the ER-targeting sequence coding region. (C) NA^{WSN} was transfected alone or with $NS1^{WSN}$ in 293T cells. 43 h after transfection, cells were treated with increasing amounts of the protease inhibitor MG132. 48 h after transfection, the cells were harvested, and NA levels were analyzed by activity and immunoblotting. (D) 293T cells were transfected with the indicated constructs for 48 h, and potential unfolded protein response induction was analyzed by eIF2 phosphorylation and splicing of XBP1 mRNA. eIF2 was analyzed by immunoblotting for total protein levels (rabbit anti-eIF2; D7D3; Cell Signaling Technology) and phosphorylated eIF2 levels (rabbit anti–phospho-eIF2α; Ser51; 119A11; Cell Signaling Technology). The portion of spliced XBP1 mRNA was analyzed by PCR with primers upstream and downstream of the splicing region. (E) 293T cells were transfected with NA^{WSN} alone or cotransfected with NS1 and plated at the indicated densities. Cells were diluted in media so that each sample had identical ratios of transfection reagent to DNA amount to cell number. 48 h after transfection, cells were harvested, and NA activity was measured. (F) Cells transfected with expression plasmids for NA, or NA and NS1 were cultured 48 h at the indicated temperatures. NA protein levels were monitored using immunoblots shown above the amido black–stained membrane (total protein). (G) Immunoblots of M2 and $_{5'\text{NA-UTR}}$M2 expressed alone or with NS1 in cells at 40°C for 48 h.

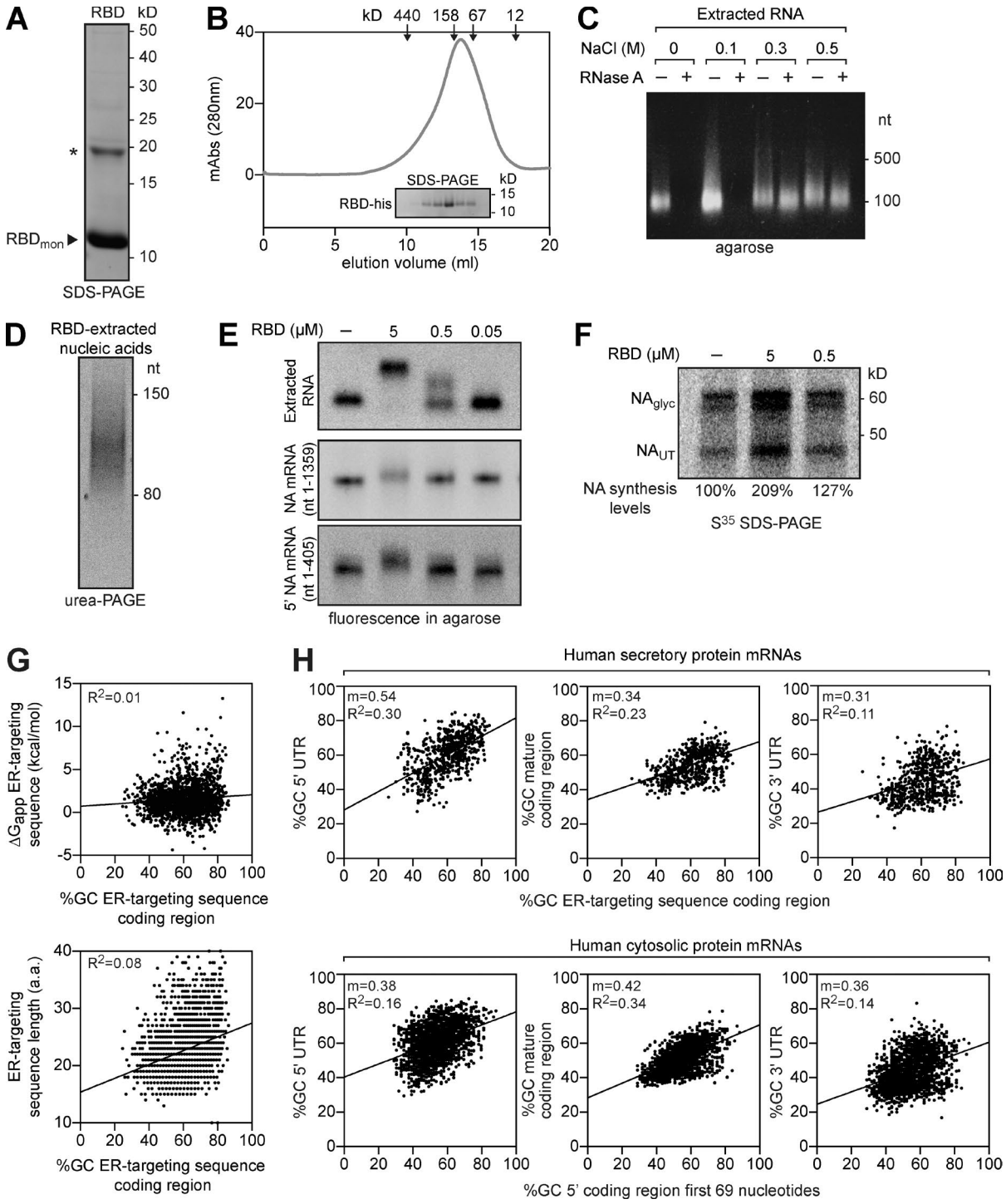

Figure S3. **In vitro analysis of the NS1 RBD and the GC content analysis of human mRNAs.** (A) Coomassie-stained gel of the RBD (5 µg) purified from *E. coli*. The SDS-resistant RBD dimer is indicated with an asterisk. (B) Size-exclusion chromatography profile of the purified RBD is shown with the indicated molecular weight standards and a Coomassie-stained gel of the corresponding peak fractions. (C) 200 ng of RNA extracted from the RBD was treated with 1 µg RNase A for 30 min at indicated NaCl concentrations. The products were resolved on 2% ethidium bromide agarose. At NaCl concentrations ≥0.3 M, RNase A only cleaved single-stranded RNA. (D) The RBD-bound RNA fragments were resolved on a denaturing urea-PAGE gel and stained using SYBR green. (E) Equal amounts of FITC-labeled NS1 extracted RNA and the indicated in vitro transcribed NA mRNAs were incubated 10 min with increasing RBD concentrations, complexes were separated on a 1% agarose gel, and the labeled RNA was visualized. (F) NA mRNA was in vitro translated in the presence of microsomes and the indicated RBD concentration for 30 min at 30°C. Translocated and fully glycosylated NA is denoted above the untranslocated NA. The translation level in the absence of the RBD was set to 100%. (G) The GC content in ER-targeting sequence coding regions from human-secreted soluble proteins does not correlate to properties in the resulting peptide (top, hydrophobicity determined by the ΔG_{app} of membrane insertion; bottom, amino acid length). (H) Correlation plots comparing the GC content in the indicated regions of human mRNAs encoding secreted (top) and cytosolic proteins (bottom) with 5′ UTRs of >300 nucleotides. For each mRNA, the GC content in the 5′ coding region (ER-targeting sequences of secreted proteins or the first 69 nucleotides of cytosolic proteins) was plotted with respect to the 5′ UTR (left), protein coding region (middle), and 3′ UTR (right). Pearson's correlation coefficient (R^2) is shown with the slope (m) of each linear regression curve.