Molecular Cell, *Volume 45*

Supplemental Information

Immobilization of Proteins in the Nucleolus by Ribosomal Intergenic Spacer Noncoding RNA

Timothy E. Audas, Mathieu D. Jacob, and Stephen Lee

Supplemental Experimental Procedures

Plasmids and Adenovirus Vectors

The adenovirus and plasmids expressing FLAG-VHL-GFP, APC2-GFP, RNF8-GFP, POLD1-GFP and Hsp110-GFP, were described previously (Mekhail et al., 2007). pHsp70-GFP and pHsp70v-GFP (a.a.161-496) were cloned from MCF-7 genomic DNA and fused to a carboxyl-terminal GFP in pcDNA3.1. The pFLAG-recNoDS-GFP plasmid was cloned using synthesized oligonucleotides (Qiagen) ligated into a plasmid downstream of a FLAG-tag and upstream of the GFP fusion protein. pGEM-IGS_{28} and $pcIGS_{28}$ were cloned from MCF-7 genomic DNA into pGEM-T-Easy (Promega) and pcDNA3.1 respectively using the primers 5'-CTGCCCTTCCACGAGAGTG and 5'-CCGAGTCTCACTCACTCTACA. Plasmids containing IGS_{22}RNA were PCR amplified from MCF-7 genomic DNA and total RNA using the primers 5'-AACACCACCTCCTTGACCTG and 5'-ACAGAGAGAAGGCCCTAGCC and cloned *Hind*III/*Xho*I (Fermentas) into pcDNA3.1. The mutants VHLΔRM (1-170(Δ107-120)), Hsp70ΔRM (1-641(Δ261-267)) and Hsp70ΔRM (161-496(Δ261-267)) were made using the Stratagene Quikchange site-directed mutagenesis kit. The RNF8 arginine domains mutant (RNF8ΔRM) is missing the amino and carboxyl terminal and contains the cDNA sequence encoding amino acids 25-468 fused to GFP. The plasmids B23-GFP (M. Olson; University of Mississippi Medical Center, Jackson, MS), MDM2-GFP (G. Pei; Shanghai Institute of Biological Sciences, Shanghai, China), GFP-DNMT1 (K. Robertson; University of Florida, Shands Cancer Center, Gainesville, FL) and GFP-PABP1 (N. Kedersha; Harvard Medical School, Boston, MA) were generously provided. MDM2-GFP mutants were produced by PCR amplification of the cDNA sequence corresponding to the given amino acid sequences of MDM2. These products were cloned upstream of the GFP open reading frame inserted into pcDNA3.1. The following plasmids were obtained from Addgene and the cDNA sequences were subcloned into pEGFP-C_2 (Clontech) where necessary: pBABE HSF Flag wt (R. Kingston: Addgene plasmid 1948), HA-cdh1 (M. Kirchner (Pfleger et al., 2001): Addgene plasmid 11596), YF-Tiam1 (T. Meyer (Inoue et al., 2005): Addgene plasmid 20154), F-DAXX/pRK5 (X. Yang (Tang et al., 2006): Addgene plasmid 27974), pWZL Neo Myr Flag PRKAA1 (J. Zhao (Boehm et al., 2007): Addgene plasmid 20595), pEGFP-C1-hUVRAG (N. Mizushima (Itakura et al., 2008): Addgene plasmid 24296), pBABE HSF Flag wt (R. Kingston (Sullivan et al., 2001): Addgene plasmid 1948),

pQCXIP Flag-hRpt6 (R. Conaway (Yao et al., 2008): Addgene plasmid 19793) and pCMV flag ERR alpha (T. Finkel (Ichida et al., 2002): Addgene plasmid 10975)

Tryptic Cleavage Assay

Proteins were extracted by sonicating untreated and acidified MCF-7 cells in lysis buffer without the addition of protease inhibitors and quantified using the BCA protein assay (Pierce Biotechnology Inc., Rockford, IL) to normalize protein concentrations. Aliquots of the acidified lysates were subsequently treated for 30 min with RNase A or DNase I at 37°C. Equal amounts of protein were next exposed to 0, 5, 10 or 20ng of trypsin for 30 min at 37°C. Following cleavage proteins were separated by 1D SDS-PAGE on a 10% gel and transferred to PVDF membrane. Blots were probed with primary antibodies: M2 at 1:2000 and visualized using ECL Plus (GE Healthcare) on x-ray film.

Analysis of Nucleolar Localization

Heat shock and acidosis treated MCF-7 cells were harvest and fixed at the indicated time points, prior to direct visualization of exogenous VHL-GFP or indirect immunostaining of endogenous Hsp70. The proportion of cells possessing nucleolar VHL-GFP was calculated by identifying the presence or absence of a nucleolar localization, in triplicate, of 100 transfected cells. Endogenous Hsp70 localization was calculated by counting at least 3 fields and dividing nucleolar Hsp70 sequestered cells by the total cell number using Hoechst counterstaining.

Relative Transcript Count

Standard curves were created by supplementing 3μg of *Escherichia coli* total RNA (Ambion) with $1.5x10^8$, $1x10^8$, $5x10^7$, $2x10^7$, $1x10^7$ and $5x10^6$ copies of *in vitro* transcribed $IGS_{22}RNA/IGS_{28}RNA$ or $3x10^9$, $2x10^9$, $1x10^9$, $5x10^8$, $1x10^8$ and $5x10^7$ copies of *in vitro* transcribed β-actin. Next a reverse transcriptase reaction (High Capacity cDNA synthesis Kit-Applied Bioscience) was simultaneously performed using random primers on *E. coli* RNA, supplemented as above, and RNA extracted from heat shocked or acidotic MCF-7 cells. Quantitative PCR (qPCR) analysis was carried out using a SYBR Green PCR Master Mix (Applied Biosystems) and samples were analyzed on an ABI 7300 system. Standard curves were generated and analyzed using the ABI 7300 System Sequence Detection Software (v. 1.2.2) and Microsoft Excel.

Cell Viability Assay

Unfixed MCF-7 cells transfected with pcDNA3.1, pGEM-IGS_{28} or $pcIGS_{28}$ were co-stained with Hoechst (33342), propidium iodide (PI) and fluorescein diacetate (FDA). At least three fields were counted at 20x magnification and the ratio of living and dead cells were calculated as FDA positive divided by Hoechst positive and PI positive divided by Hoechst positive cells, respectively.

Primers for ChIP and RT-PCR

IGS_{28}RNA Mapping	Set A	5'-TCTCTCGCTGTCCATCTCTG	5'-GACCTCCCGAAATCGTACAC
	Set B	5'-TCTGTCTCTTTCTCTGTCAGTCTT	5'-GAAAATGCCCCTCGCATC
	Set C	5'-CCTTCCACGAGAGTGAGAAG	5'-CGGGGAAATAGGAGAAGTACG
	Set D	5'-GTGCTTCGGTGCTTAGAGAG	5'-CAGTTTATGTTGAAGTCGAGGA
	Set E	5'-GGCCGAGAGGAATCTAGACA	5'-CGTCCGGCCTTAACAGTTT
	Set F	5'-GATGCGAGGGGCATTTTC	5'-CCGAGGAGCTGGCATTAC
	Set G	5'-GTGTGGCGTCCGTACTTCTC	5'- CCGCTTCCCTGGTTCAAG
	Set H	5'-TCTCCTCGACTTCAACATAAACT	5'-CTGGGCCGTAGTGCAGTG
	Set I	5'-GCGAAACCCCGTCTCTACTA	5'-TTCCGTATTTATTTAGAGACCGA
	Set J	5'-AGCTGAGTCGGGAGCGG	5'-CAGCAGGGAAAAGAATTAATGA
	Set K	5'-AGGCTGAGGCGGGAGAAT	5'-TAGGTGATGCCCGAAGACA
	Set L	5'-GCCCAGGCTGTAGAGTGAGT	5'-CCCCAGGCTCGGTGAAG
	Set M	5'-CGGTCTCTAAATAAATACGGAAA	5'-CCTCCAGAAGGGAGAGAGAGA
	Set N	5'-TAATTCATTAATTCTTTTCCCTGC	5'-CTAGGCAACGAGGGAGAGAG
ChIP RT-PCR	Pre-rRNA	5'-GGCGGTTTGAGTGAGACGAGA	5'-ACGTGCGCTCACCGAGAGCAG
	$rRNA_{04}$	5'-CGACGACCCATTCGAACGTCT	5'-CTCTCCGGAATCGAACCCTGA
	$rRNA_{08}$	5'-AGTCGGGTTGCTTGGGAATGC	5'-CCCTTACGGTACTTGTTGACT
	$rRNA_{12}$	5'-GAGCTCAGGGAGGACAGAAA	5'-AGGTCAGAAGGATCGTGAGG
	IGS_{16}	5'-ACACACACACACCCCGTAGT	5'-GAAATGGGGCTTCGATACAT
	IGS_{18}	5'-GTTGACGTACAGGGTGGACTG	5'-GGAAGTTGTCTTCACGCCTGA
	IGS_{20}	5'-GTAGCCTTGGGCTTCTCTCC	5'-AGTTTTCAGCCCCAACACAC
	IGS_{22}	5'-CAGTGGCTCACGTCTGTCAT	5'-CGCCTGACTCCATTTCGTAT
	IGS_{24}	5'-CCCGCGCACATAATAACTAA	5'-AAATCACTCCTCACGGGAAC
	IGS_{28}	5'-CCTTCCACGAGAGTGAGAAG	5'-GACCTCCCGAAATCGTACAC
	IGS_{30}	5'-GGTCTCTGCGTCTCGCTATC	5'-TGAAGAATTCAGGCCTTGGT
	IGS_{32}	5'-AAAAGCTGGCCGATCTGAAT	5'-CGTCTGTTCAGCTATTTTGCAG
	IGS_{34}	5'-CCATGCCTTCGACTCTGTAA	5'-GTACTGTGCCAAATCGGAAA
	IGS_{36}	5'-TCCACTCCCAAGTTCAGTGG	5'-CGAGGGAACCCAAGGTAGAG
	IGS_{38}	5'-CTCACAGAGGAAGGGAGCAC	5'-AACAAGGGAGGGAGGAACTT
	IGS_{40}	5'-TTCTCCTTGGTCAGGGGTTT	5'-CAGGAAAGTCCCCAACAACA
	IGS_{42}	5'-GCTTCTCGACTCACGGTTTC	5'-CCGAGAGCACGATCTCAAAG
FISH	IGS_{28}-anti-sense	5'-DIG-CCGGCCTTAACAGTTTATGTTGAAGTCGAGGAGACTTATCGGGGAAATAGGAGAAGTACG-DIG	
	IGS_{28}-sense	5'-DIG-CGTACTTCTCCTATTTCCCCGATAAGTCTCCTCGACTTCAACATAAACTGTTAAGGCCGG-DIG	
	IGS_{28}#2	5'-DIG- CAGCCTCCCGAGGAGCTGGCATTACAGGGCCTGCCCCACCGCTCCCGACTCAGCTTTGTA-DIG	
	IGS_{28}#2 (no DIG)	5'- CAGCCTCCCGAGGAGCTGGCATTACAGGGCCTGCCCCACCGCTCCCGACTCAGCTTTGTA	

Supplemental References

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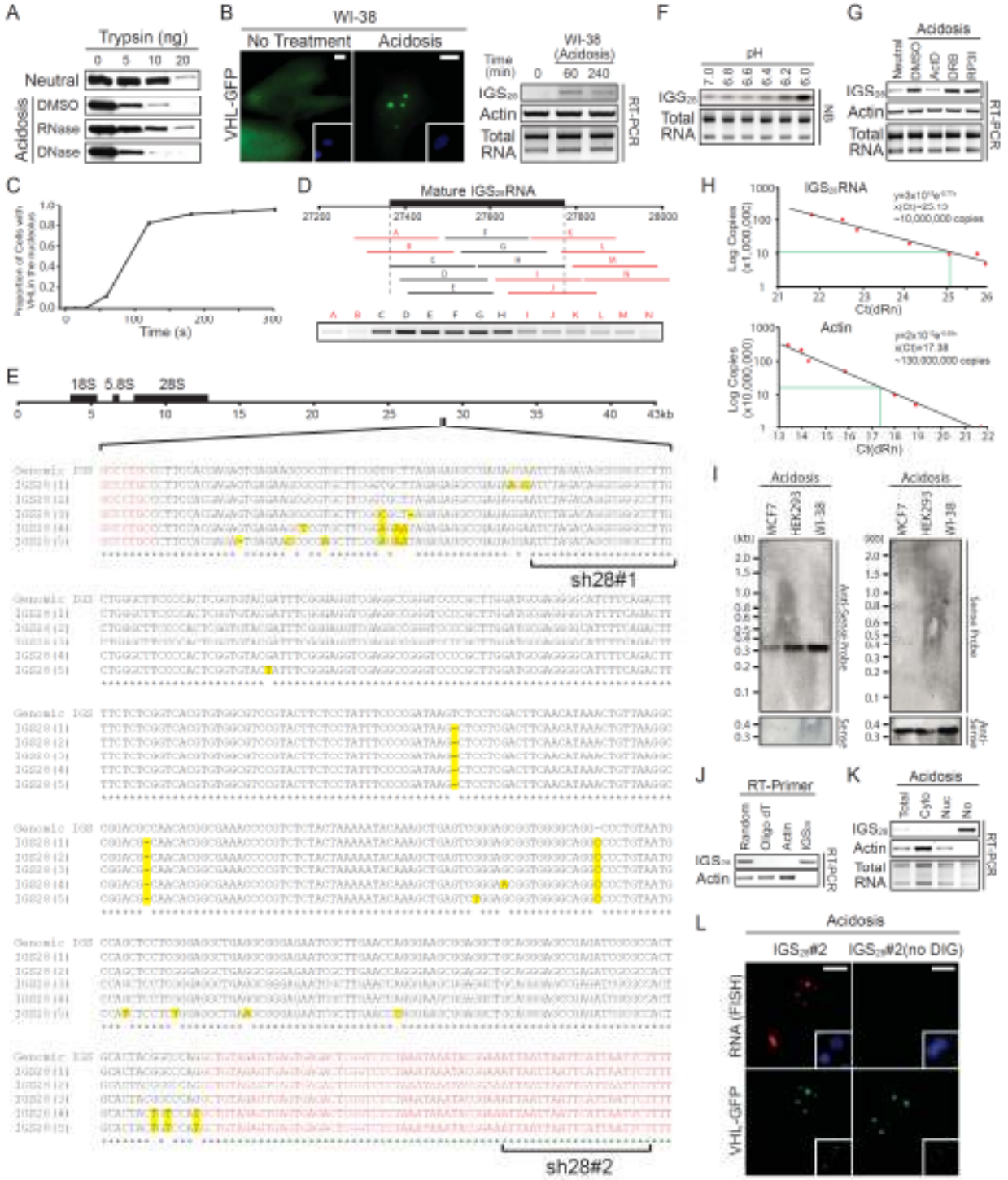
Yao, T., Song, L., Jin, J., Cai, Y., Takahashi, H., Swanson, S.K., Washburn, M.P., Florens, L., Conaway, R.C., Cohen, R.E., and Conaway, J.W. (2008). Distinct modes of regulation of the Uch37 deubiquitinating enzyme in the proteasome and in the Ino80 chromatin-remodeling complex. Molecular cell *31*, 909-917.

Figure S1


A
Trypsin (ng)
0 5 10 20
Neutral
Acidosis
DMSO
RNase
DNase
B
WI-38
No Treatment
Acidosis
VHL-GFP
WI-38 (Acidosis)
Time (min)
0 60 240
IGS28
Actin
Total RNA
RT-PCR
F
pH
7.0 6.8 6.6 6.4 6.2 6.0
IGS28
Total RNA
NB
G
Acidosis
Neutral
DMSO
ActD
DRB
BrPDI
IGS28
Actin
Total RNA
RT-PCR
C
Time (s)
D
Mature IGS28RNA
27200 27400 27600 27800 28000
A B C D E F G H I J K L M N
E
18S 5.8S 28S
0 5 10 15 20 25 30 35 40 43kb
sh28#1
sh28#2
H
IGS28RNA
Log Copies (x1,000,000)
Ct(dRn)
~10,000,000 copies
Actin
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~130,000,000 copies
I
Acidosis
MCF7
HEK293
WI-38
Anti-Sense Probe
Sense Probe
Sense
Anti-Sense
J
RT-Primer
Random
Oligo dT
Actin
IGS28
RT-PCR
K
Acidosis
Total
Cyto
Nuc
No
IGS28
Actin
Total RNA
RT-PCR
L
Acidosis
IGS28#2
IGS28#2(no DIG)
RNA (FISH)
VHL-GFP

Figure S1. Supplemental Characterization of the IGS_{28}RNA Transcript, Related to Figure 1

(A) RNA mediates changes in protein conformation in response to acidosis. Lysates from MCF-7 cells grown under standard or acidotic conditions were cleaved with the indicated amount of trypsin, following an RNase or DNase digestion where indicated. Cleaved VHL-GFP was detected by western blotting.

(B) WI-38 cells are responsive to low pH. WI-38 cells were infected with an adenovirus-expressing VHL-GFP and left untreated or exposed to acidosis prior to visualization. Hoechst staining was inset (left panel). RNA from untreated or acidotic WI-38 cells was extracted and IGS_{28}RNA and β-actin were amplified by RT-PCR (right panel). Bars, 10 μm.

(C) VHL-GFP sequestration occurs within two hours of acidosis treatment. MCF-7 cells expressing VHL-GFP were grown in pH 6.0 media and visualized at 0, 15, 30, 60, 120, 180, 240 and 300 minutes post-treatment to assess the proportion of cells containing VHL localized to the nucleolus. Data was collected in triplicate.

(D) RT-PCR mapping of the IGS_{28}RNA. Schematic diagram of the IGS_{28} loci with the positions of the PCR amplicons used for RT-PCR analysis indicated below (upper panel). RNA from acidotic MCF-7 cells was extracted and analyzed by RT-PCR using primers approximately every 30 nucleotides to produce 200 base pair amplicons (lower panel).

(E) IGS_{28}RNA possesses multiple polymorphisms. Schematic diagram of the 43kb human rDNA cassette (upper panel). The pre-mature IGS_{28}RNA transcript was amplified with a high fidelity Taq polymerase from RT product and inserted into the pGEM-T-easy plasmid. Five independent clones were analyzed and compared to the GenBank sequence (U13669). The positions of the shRNAs are indicated. Red font corresponds to the pre-mature transcript (lower panel).

(F) IGS_{28}RNA is induced by low pH. Northern blot of IGS_{28}RNA from MCF-7 cells allowed to acidify to the indicated pH.

(G) IGS_{28}RNA induction is regulated by RNA Polymerase I. MCF-7 cells were pre-treated for 1 hour with DMSO, 0.1μM Actinomycin D (ActD), 50μM 5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole (DRB) or 25μM RNA Polymerase III Inhibitor (RP3I), followed by

a 1 hour acidosis treatment, with inhibitors. RNA levels were measured by semi-quantitative RT-PCR.

(H) IGS_{28}RNA is moderately expressed when compared to β-actin. Quantitative PCR analysis of mature IGS_{28}RNA and β-actin transcripts from acidotic MCF-7 cells. Standard curves were created by supplementing *E. coli* RNA with a specific number of copies of IGS_{28}RNA and β-actin transcripts, prior to the reverse transcription and qPCR.

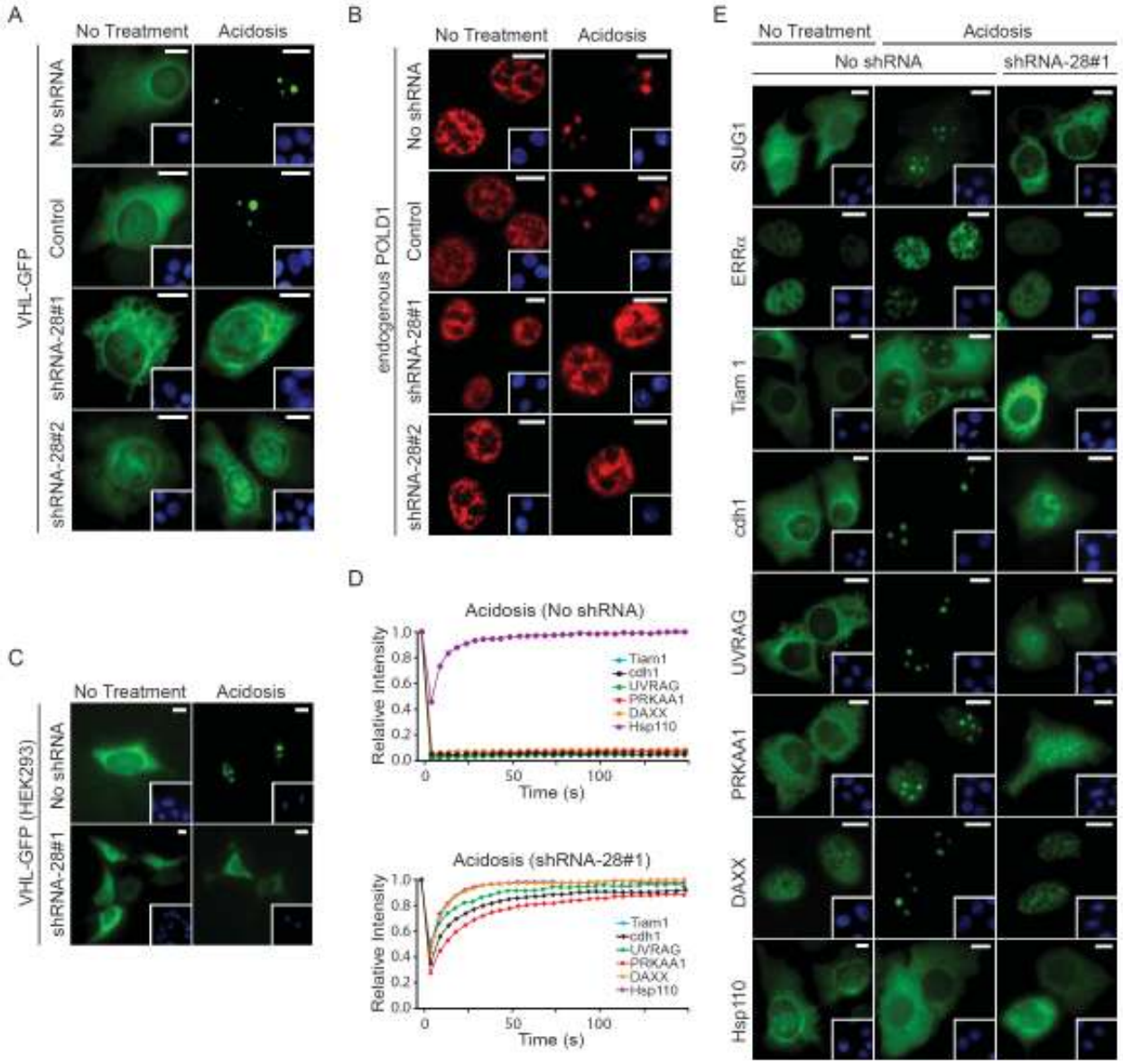
(I) IGS_{28}RNA is expressed from the same strand as rRNA. Northern blots of RNA extracted from MCF-7, HEK293 and WI-38 cells grown in pH 6.0 media for 4 hours using sense and anti-sense probes. Upper membranes were stripped and re-probed with the complementary probe and included below.

(J) IGS_{28}RNA is not polyadenylated. A reverse transcription reaction was performed using random, oligo dT, β-actin-specific or IGS_{28}-specific primers prior to semi-quantitative RT-PCR.

(K) IGS_{28}RNA is localized to the nucleolus. Acidified MCF-7 cells were separated into total, cytoplasmic (Cyto), nuclear (Nuc) and nucleolar (No) RNA fractions for semi-quantitative RT-PCR of IGS_{28}RNA. β-actin and total RNA were included as controls.

(L) IGS_{28}RNA and VHL-GFP co-localize to the nucleolus during acidosis. MCF-7 cells transfected with VHL-GFP were acidified and IGS_{28}RNA was detected by fluorescent *in situ* hybridization using digoxigenin-labeled or unlabeled oligonucleotide probes (red). Hoechst (blue) and GFP/RNA overlay are inset.

Figure S2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No Treatment
Acidosis
VHL-GFP
No shRNA
Control
shRNA-28#1
shRNA-28#2
B
No Treatment
Acidosis
endogenous POLD1
No shRNA
Control
shRNA-28#1
shRNA-28#2
C
No Treatment
Acidosis
VHL-GFP (HEK293)
No shRNA
shRNA-28#1
D
Acidosis (No shRNA)
Relative Intensity
Time (s)
Tiam1
cdh1
UVRAG
PRKAA1
DAXX
Hsp110
Acidosis (shRNA-28#1)
Relative Intensity
Time (s)
Tiam1
cdh1
UVRAG
PRKAA1
DAXX
Hsp110
E
No Treatment
Acidosis
No shRNA
shRNA-28#1
SUG1
ERRα
Tiam 1
cdh1
UVRAG
PRKAA1
DAXX
Hsp110

Figure S2. IGS_{28}RNA Regulates Nucleolar Sequestration during Acidosis, Related to Figure 2

(A and B) Knockdown of IGS_{28}RNA inhibits the nucleolar sequestration of VHL-GFP (**A**) and endogenous POLD1 (B). Untreated and acidotic MCF-7 cells expressing shRNA targeting a scramble sequence (Control), or IGS_{28} (shRNA-28#1 and shRNA-28#2) were visualized for adenovirus-expressed VHL-GFP and endogenous POLD1 with Hoechst inset. Bars, 10 µm.

(C) Stable HEK293 cell lines expressing shRNA targeted to the IGS_{28}RNA were left untreated or allowed to acidify under hypoxic conditions. Adenovirus-encoded VHL-GFP was visualized by fluorescence microscopy with Hoechst nuclear stain inset. Bars, 10 µm.

(D) Quantification of fluorescence recovery after photobleaching of Tiam1-GFP, cdh1-GFP, UVRAG-GFP, PRKAA1-GFP, DAXX-GFP and Hsp110-GFP in acidotic MCF-7 and IGS_{28}RNA knockdown (shRNA-28#1) cells.

(E) NoDS-containing protein recruitment to the nucleolus is dependent on IGS_{28}RNA. MCF-7 and shRNA-28#1 cells were transfected with the NoDS-containing proteins: SUG1, ERRα, Tiam1, cdh1, UVRAG, PRKAA1 and DAXX or the non-NoDS-containing Hsp110 and exposed to acidosis. Hoechst inset (blue). Bars, 10 µm.

Figure S3

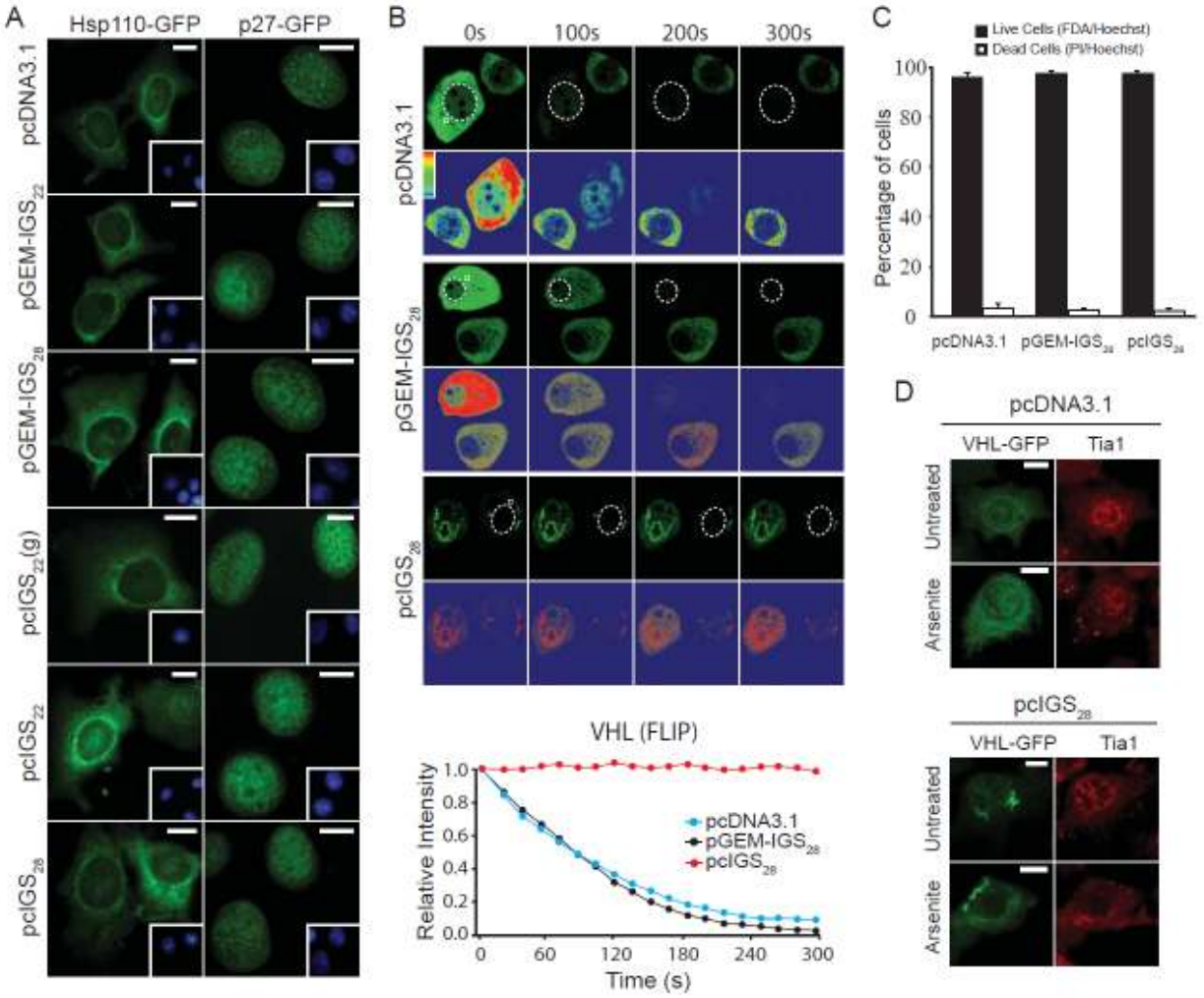

A
Hsp110-GFP
p27-GFP
pcDNA3.1
pGEM-IGS22
pGEM-IGS28
pcIGS22(g)
pcIGS22
pcIGS28
B
0s
100s
200s
300s
pcDNA3.1
pGEM-IGS28
pcIGS28
VHL (FLIP)
Relative Intensity
Time (s)
pcDNA3.1
pGEM-IGS28
pcIGS28
C
Live Cells (FDA/Hoechst)
Dead Cells (PI/Hoechst)
Percentage of cells
pcDNA3.1
pGEM-IGS24
pcIGS24
D
pcDNA3.1
VHL-GFP
Tia1
Untreated
Arsenite
pcIGS28
VHL-GFP
Tia1
Untreated
Arsenite

Figure S3. IGSRNA Mediates Cytoplasmic Immobilization of VHL-GFP but Not the Unrelated Proteins p27 and Hsp110, Related to Figures 3 and 5

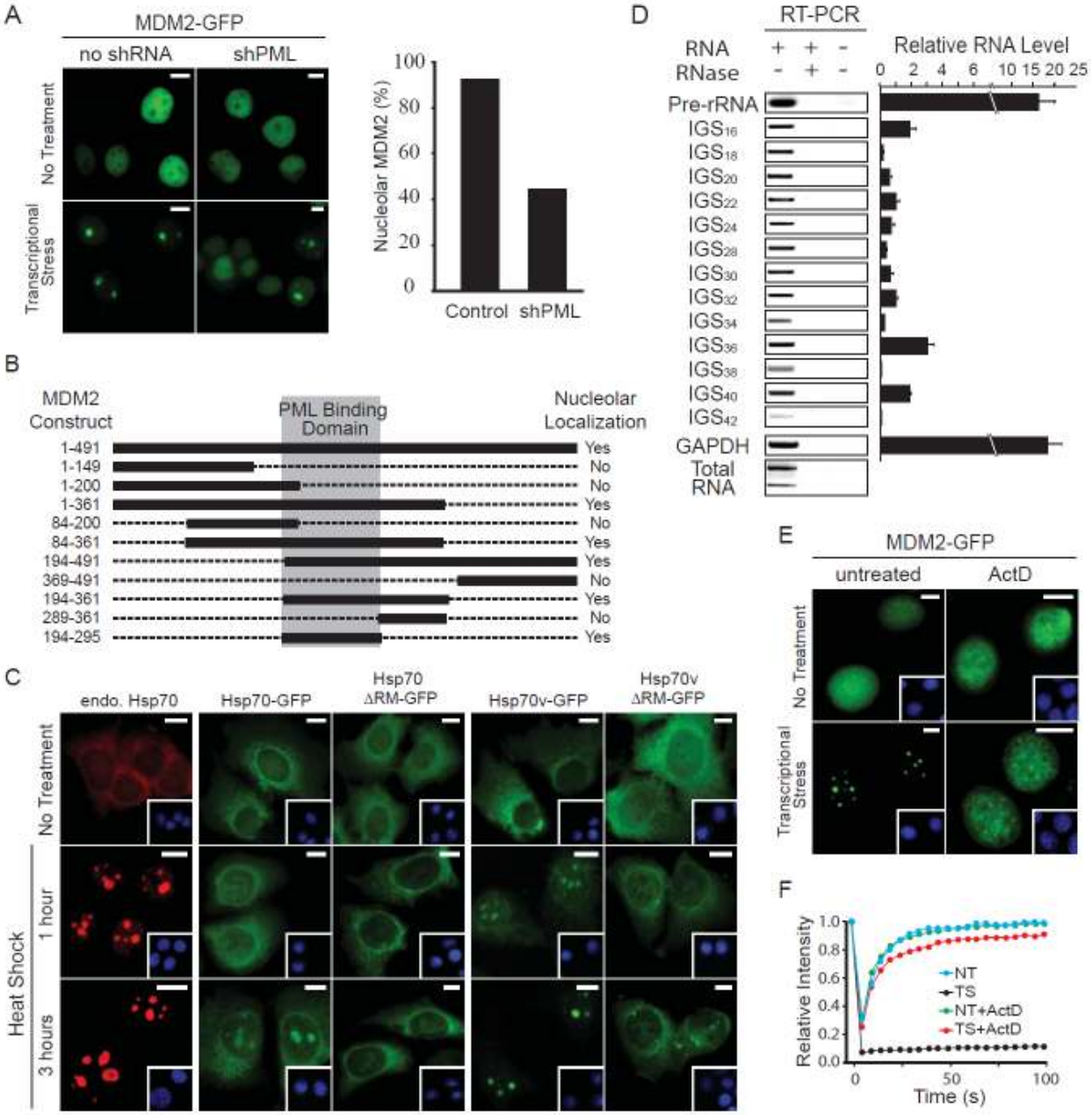
(**A**) Hsp110 and p27 localization is unaffected by IGSRNA. MCF-7 cells were transfected with plasmids encoding p27-GFP or Hsp110-GFP and the effector constructs pcDNA3.1, pGEM-IGS_{22}, pGEM-IGS_{28}, $pcIGS_{22}$(g), $pcIGS_{22}$ or $pcIGS_{28}$. Cells were monitored for IGSRNA-mediated aggregation by fluorescence microscopy, with Hoechst inset. Bars, 10 µm.

(B) FLIP analysis of VHL-GFP mobility in cells co-transfected with pcDNA3.1, pGEM-IGS_{28} or $pcIGS_{28}$. Transfected MCF-7 cells were submitted to repeated bleaching of the indicated regions (square) outside of the nucleus (dashed circle) and imaged between pulses at the indicated times (upper panel). Quantification of the loss of fluorescence of VHL-GFP with pcDNA3.1, pGEM-IGS_{28} or $pcIGS_{28}$ (lower panel).

(C) Over-expression of IGS_{28}RNA does not lead to increased cell death. MCF-7 cells transfected with pcDNA3.1, pGEM-IGS_{28} or $pcIGS_{28}$ were stained with the live cell dye fluorescein diacetate (FDA) or the dead cell marker propidium iodide (PI) and counterstained with Hoechst. Each error bar represents standard error of the mean of the proportion of living or dead cells from three repeats.

(D) VHL-GFP cytoplasmic aggregates are distinct from cytoplasmic stress granules. MCF-7 cells transfected with VHL-GFP and pcDNA3.1 or $pcIGS_{28}$ were left untreated or exposed to 0.5mM sodium arsenite for 30 min. Fixed cells were stained for endogenous Tia1 as a stress granule marker. Bars, 10 µm.

Figure S4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MDM2-GFP
no shRNA
shPML
No Treatment
Transcriptional Stress
Nucleolar MDM2 (%)
100
80
60
40
20
0
Control
shPML
B
MDM2 Construct
PML Binding Domain
Nucleolar Localization
1-491 Yes
1-149 No
1-200 No
1-361 Yes
84-200 No
84-361 Yes
194-491 Yes
369-491 No
194-361 Yes
289-361 No
194-295 Yes
C
endo. Hsp70
Hsp70-GFP
Hsp70 ΔRM-GFP
Hsp70v-GFP
Hsp70v ΔRM-GFP
No Treatment
Heat Shock
1 hour
3 hours
D
RT-PCR
RNA
RNase
Relative RNA Level
0 2 4 6 10 15 20 25
Pre-rRNA
IGS16
IGS18
IGS20
IGS22
IGS24
IGS28
IGS30
IGS32
IGS34
IGS36
IGS38
IGS40
IGS42
GAPDH
Total RNA
E
MDM2-GFP
untreated
ActD
No Treatment
Transcriptional Stress
F
Relative Intensity
1.0
0.8
0.6
0.4
0.2
0.0
NT
TS
NT+ActD
TS+ActD
0
50
100
Time (s)

Figure S4. Supplemental Characterization of Hsp70 and MDM2 during Heat Shock and Transcriptional Stress, Related to Figures 4 and 5

(A) Localization of MDM2-GFP in PML knockdown cells. MCF-7 cells were transfected with MDM2-GFP and an shRNA targeting PML. Cells were exposed to transcriptional stress and representative images were taken by fluorescence microscopy (left panel). The proportion of cells containing nucleolar MDM2 was calculated for control and shPML transfected cells (right panel). Bars, 10 μm.

(B) Nucleolar targeting of MDM2 requires PML binding. Deletion mutants of MDM2-GFP were constructed with the corresponding amino acid sequences, the PML binding site annotated. Nucleolar localization of the mutants during transcriptional stress was detected in transfected MCF-7 cells and summarized to the right.

(C) Exogenous Hsp70 does not target to the nucleolus as efficiently as the endogenous molecule or the truncated variant Hsp70v. Hsp70-GFP, Hsp70ΔRM-GFP, Hsp70v-GFP and Hsp70vΔRM-GFP were transfected into MCF-7 cells and heat shocked for 0, 1 or 3 hours. The localization of the exogenous molecules was compared to immunostained endogenous Hsp70.

(D) The ribosomal IGS produces multiple transcripts. High-cycle semi-quantitative RT-PCR (left) and quantitative PCR (right) of untreated (-) or RNase digested (+) RNA extracted from untreated cells was performed for the IGS transcripts with GAPDH as a control.

(E and F) General transcription inhibition blocks MDM2 nucleolar sequestration in response to transcriptional stress. (E) MCF-7 cells were left untreated or pre-treated for two hours with 8μM ActD to repress transcription. Following a 3 hour stress treatment (4μM ActD + 8μM MG132) cells were visualized. Bars, 10 μm. (F) Quantification of the fluorescence recovery of MDM2-GFP after no or ActD pre-treatment, prior to induction of transcriptional stress.

Figure S5

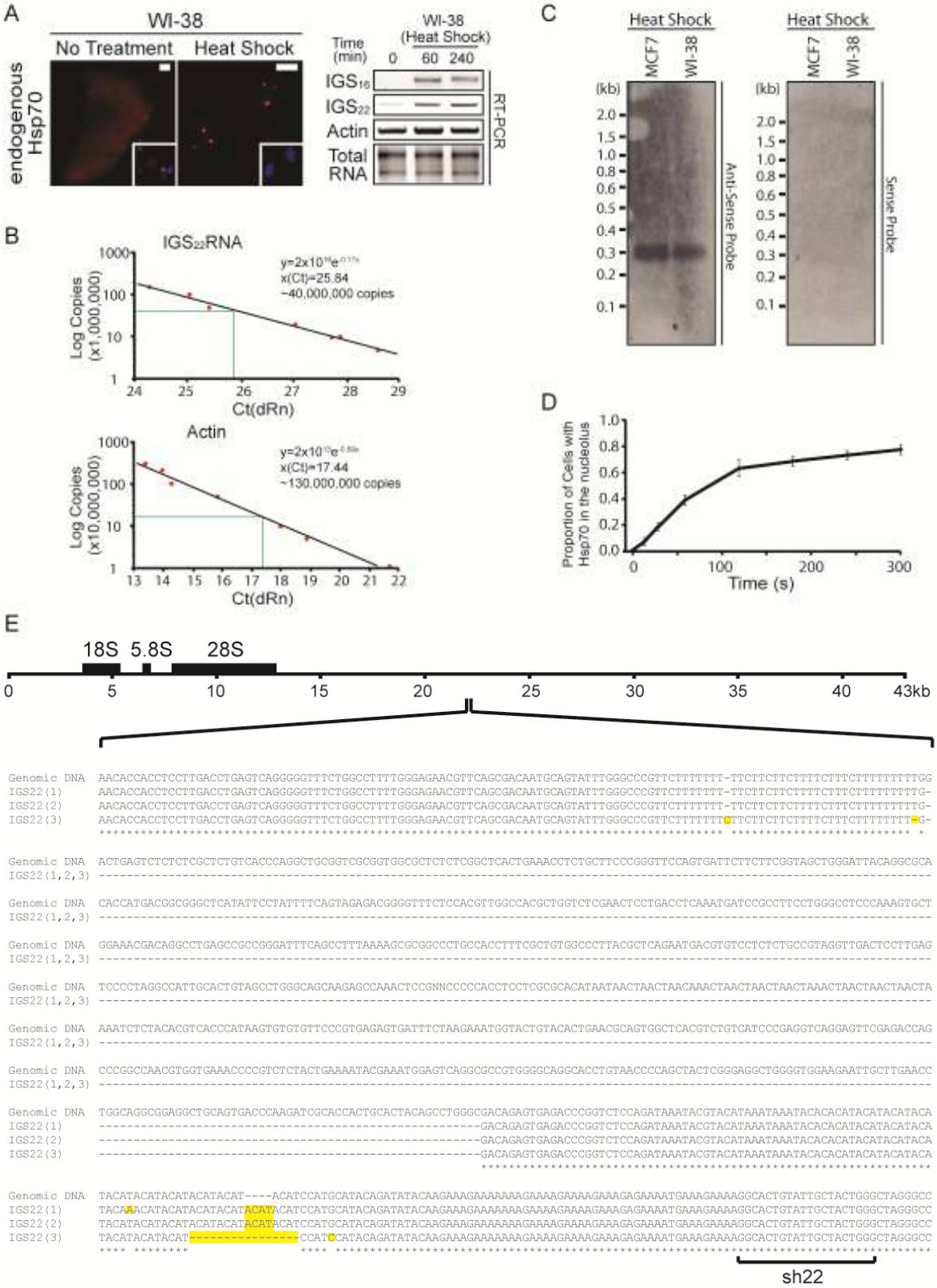
A
WI-38
No Treatment
Heat Shock
endogenous Hsp70
WI-38 (Heat Shock)
Time (min)
0
60
240
IGS16
IGS22
Actin
Total RNA
RT-PCR
B
IGS22RNA
y=2x10^16e^-0.17x
x(Ct)=25.84
~40,000,000 copies
Log Copies (x1,000,000)
Ct(dRn)
Actin
y=2x10^15e^-0.59x
x(Ct)=17.44
~130,000,000 copies
Log Copies (x10,000,000)
Ct(dRn)
C
Heat Shock
MCF7
WI-38
(kb)
Anti-Sense Probe
Sense Probe
D
Proportion of Cells with Hsp70 in the nucleolus
Time (s)
E
18S
5.8S
28S
0
5
10
15
20
25
30
35
40
43kb
Genomic DNA
IGS22(1)
IGS22(2)
IGS22(3)
IGS22(1,2,3)
sh22

Figure S5. Characterization of $IGS_{22}RNA$, Related to Figure 5

(A) WI-38 cells are responsive to heat shock. Untreated or 1 hour heat shock treated WI-38 cells were stained for endogenous Hsp70, with Hoechst staining inset (left panel). RNA from these cells was extracted and $IGS_{16}RNA/IGS_{22}RNA$ was amplified by RT-PCR (right panel). Bars, 10 μm.

(B) $IGS_{22}RNA$ is expressed at moderate levels compared to β-actin. Heat shock treated MCF-7 cells were analyzed by quantitative PCR for processed $IGS_{22}RNA$ and β-actin transcripts. Standard curves were created by supplementing *E. coli* RNA with a specific number of copies of $IGS_{22}RNA$ and β-actin transcripts, prior to the reverse transcription and qPCR.

(C) $IGS_{22}RNA$ is expressed from the rRNA sense strand. RNA extracted from MCF-7 and WI-38 cells heat shock treated for 4 hour was probed by northern blotting for $IGS_{22}RNA$ using a sense and anti-sense probe.

(D) Heat shock treatment rapidly induces Hsp70 nucleolar sequestration. MCF-7 cells were transferred to $42^{o}C$ and fix at 0, 15, 30, 60, 120, 180, 240 and 300 minutes post-treatment. Cells were stained for endogenous Hsp70 and the proportion of cells containing nucleolar Hsp70 was determined. Data was collected in triplicate.

(E) $IGS_{22}RNA$ is processed and contains several polymorphisms. Schematic diagram of the 43kb human rDNA cassette (upper panel). $IGS_{22}RNA$ was amplified with a high fidelity Taq polymerase from RT product and cloned into the pGEM-T-easy plasmid. Three independent clones were analyzed and compared to the GenBank sequence (U13669). The position of the $shIGS_{22}RNA$ (shRNA-22) target site is indicated (lower panel).

Figure S6

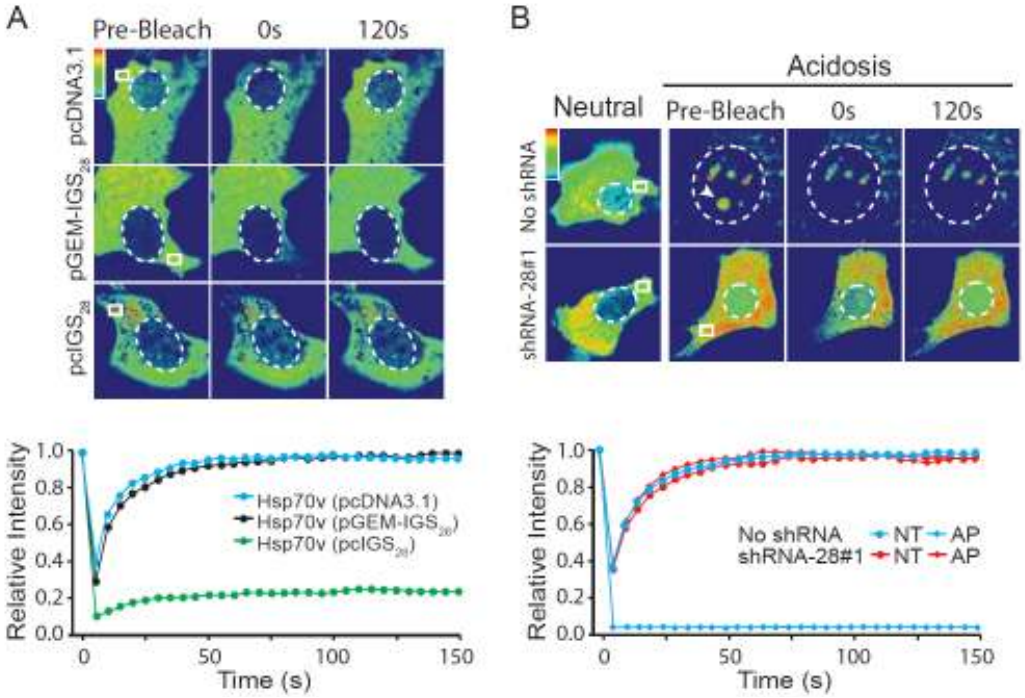

Figure S6. Hsp70 Mobility Is Affected by the IGS_{28}RNA, Related to Figure 6

(A) Hsp70v-GFP forms IGS_{28}RNA-mediated immobile aggregates. MCF-7 cells transfected with pcDNA3.1, pGEM-IGS_{28} or pcIGS_{28} were bleached in the indicated regions (squares) on the representative pseudocolor images with high (red) to low (blue) intensity provided (upper panel). Quantification of the FRAP results are the mean of 5 datasets (lower panel).

(B) Mobility of Hsp70v-GFP under neutral and acidotic conditions in shRNA knockdown cell lines. MCF-7 and shRNA-28#1 cells were bleached in the indicated cytoplasmic regions (squares) of nucleoli (arrowhead) and allowed to recover for the indicated times on the pseudocolor images (upper panel). Quantification as the mean relative intensity of 5 datasets is included (lower panel).

Figure S7

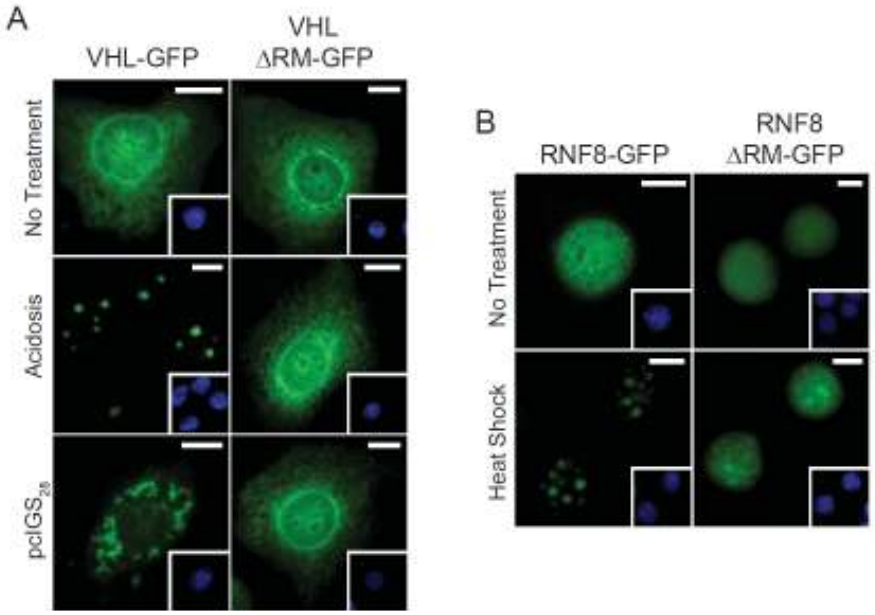

Figure S7. Deletion of the Arginine Domains of VHL and RNF8 Abrogates Protein Sequestration, Related to Figure 7

(A) Fluorescent images of VHL-GFP or the arginine deletion mutant (VHLΔRM-GFP) transfected into MCF-7 cells grown in acidotic media or over-expressing IGS_{28}RNA. Bars, 10 µm.

(B) RNF8-GFP and the arginine domain mutant RNF8ΔRM-GFP expressed in MCF-7 cells under normal growth conditions and following heat shock treatment. Hoechst DNA staining is inset. Bars, 10 µm.