Heat shock phenocopies E1B-55K late functions and selectively sensitizes refractory tumor cells to ONYX-015 oncolytic viral therapy

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Summary

ONYX-015 is an E1B-55K-deleted adenovirus that has promising clinical activity as a cancer therapy. However, many tumor cells fail to support ONYX-015 oncolytic replication. E1B-55K functions include p53 degradation, RNA export, and host protein shutoff. Here, we show that resistant tumor cell lines fail to provide the RNA export functions of E1B-55K necessary for ONYX-015 replication; viral 100K mRNA export is necessary for host protein shutoff. However, heat shock rescues late viral RNA export and renders refractory tumor cells permissive to ONYX-015. These data indicate that heat shock and late adenoviral RNAs may converge upon a common mechanism for their export. Moreover, these data suggest that the concomitant induction of a heat shock response could significantly improve ONYX-015 cancer therapy.

Introduction

Oncolytic viruses that undergo selective lytic replication in tumor cells have the potential to fulfill many of the aspirations of cancer therapy (Chiocca, 2002; Dobbelstein, 2004; O'Shea, 2005). dl1520/ONYX-015 is the prototype for oncolytic adenoviral therapy (Bischoff et al., 1996) and the first such genetic agent to be tested in humans (Khuri et al., 2000). ONYX-015 lacks the E1B-55K gene product for p53 degradation and therefore was predicted to selectively replicate in tumor cells in which the p53 pathway is inactive (Barker and Berk, 1987; Bischoff et al., 1996). In contrast, in primary cells, the loss of E1B-55K-mediated p53 degradation was expected to activate a p53 checkpoint that would limit ONYX-015 replication. Consistent with this, ONYX-015 replication is attenuated in primary cells but replicates to wild-type virus levels in many tumor cell lines. In addition to its role in p53 inactivation, E1B-55K has late functions that include the shutdown of host protein synthesis and the preferential export and translation of late viral mRNAs, both necessary events for a productive viral infection (Babiss et al., 1985; Leppard and Shenk, 1989; Pilder et al., 1986). Recently, it was shown that the loss of E1B-55K-mediated late functions, rather than p53 inactivation, is the major determinant of ONYX-015's oncolytic selectivity (O'Shea et al., 2004).

ONYX-015 has undergone extensive clinical testing with proven safety and evidence of promising clinical activity from several indications (Khuri et al., 2000; Kirn, 2001; Reid et al., 2002; Ries and Korn, 2002; Rudin et al., 2003). Initially, ONYX-015 was evaluated by direct intratumoral injections in head and neck patients who had failed prior chemotherapy. When ONYX-015 was combined with conventional chemotherapy, over 63% of injected tumors showed significant regression (Khuri et al., 2000). In contrast, normal cells, injected adjacent to the tumor mass, showed no adverse effects to ONYX-015 therapy. Clinical responses have also been observed in recent studies, in which ONYX-015 was injected into unresectable pancreatic carcinomas (Hecht et al., 2003) and peritumoral regions of recurrent malignant gliomas (Chiocca et al., 2004). In patients suffering from metastatic colorectal cancer, the intravascular delivery of ONYX-015 to the liver provoked tumor regressions, with evidence of prolonged viremia and fever in responsive patients (Reid et al., 2002). In addition, promising clinical efficacy was observed when ONYX-015 was administered as a mouthwash to patients suffering from premalignant oral leukoplakia (Rudin et al., 2003). Moreover, ONYX-015 has recently been "armed" with genes encoding prodrug converting enzymes (Stubdal et al., 2003) or endostatin (Li et al., 2005), which would be expected to further enhance ONYX-015's clinical activity. Taken together, ONYX-015 has a promising clinical

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S I G N I F I C A N C E

ONYX-015 is an E1B-55K mutant adenovirus that undergoes selective lytic replication in tumor cells. In clinical trials, ONYX-015 therapy provoked sustained responses in many cancer patients, but this varied considerably for unknown reasons. Here, we show that tumor cell differences in providing the late functions of E1B-55K underlie permissivity/resistance to ONYX-015. Moreover, we demonstrate that the induction of a heat shock response, by incubation at 39.4°C (the temperature of a fever), or with benzoquinoid ansamycins, phenocopies E1B-55K late functions and selectively sensitizes resistant tumor cells to ONYX-015 oncolytic replication. This has important clinical applications and suggests that agents that elicit a cellular heat shock response could have a major impact on the efficacy of ONYX-015 as a cancer therapy.

profile for the treatment of disparate tumor types. Nevertheless, to date ONYX-015 has not completed randomized clinical trials, so its objective therapeutic value is hard to assess. While many patients exhibited sustained clinical responses to ONYX-015 treatment, this varied considerably, and some patients experienced no clinical relief of their disease. Therefore, understanding the molecular basis of ONYX-015 tumor cell resistance, or alternatively, how to sensitize refractory tumor cells to ONYX-015 treatment, has important therapeutic implications.

In tissue culture, tumor cell lines also vary considerably in their ability to support ONYX-015 replication (see Table S1 in the Supplemental Data available with this article online). For example, ONYX-015 replicates to wild-type virus levels in HCT-116 colorectal cancer cells (permissive), whereas it is attenuated by almost 100-fold in U2OS osteosarcoma cells (resistant). The aim of this study was to understand the mechanism underlying tumor cell resistance to ONYX-015, with the ultimate goal of either predicting responsive tumor types and/or sensitizing refractory cancer cells to ONYX-015 therapy. Here, we show that, in contrast to permissive tumor cell lines, refractory tumor cells infected with ONYX-015 fail to shut down cellular protein synthesis in the absence of E1B-55K. We show that the shutdown of cellular protein synthesis in permissive tumor cells is a secondary consequence of 100K mRNA export, an event normally regulated by E1B-55K, and rate limiting for ONYX-015 replication. The 100K protein acts downstream of E1B-55K to inhibit the translation of capped cellular mRNAs, while viral RNAs are preferentially translated due to their possession of a unique 5′UTR (Cuesta et al., 2000b; Hayes et al., 1990). Moreover, we demonstrate that the induction of a cellular heat shock response, through physical or pharmacological means, selectively rescues the export of late viral RNAs, such as 100K, hexon, and fiber, in resistant tumor cells, rendering them competent for ONYX-015 replication. These data suggest that in adenoviral infection and a cellular heat shock response, RNA export may be similarly regulated. In addition, this study suggests that the induction of a heat shock response, by febrile temperatures or pharmacological adjuvants, would greatly enhance the clinical efficacy of ONYX-015 as a cancer therapy.

Results

Tumor cell differences in providing the host protein shutoff and RNA export functions of E1B-55K correlate with ONYX-015 permissivity/resistance

Previously, we demonstrated that the propensity of permissive tumor cells to support ONYX-015 replication correlated with their unexpected ability to shut down host protein synthesis and export late viral RNAs in the absence of E1B-55K, a propensity not shared by primary cells (O'Shea et al., 2004). Therefore, we hypothesized that the variable replication of ONYX-015 in tumor cell lines (Table S1) was likely due to tumor cell differences in providing the late functions of E1B-55K. Host/viral protein synthesis was measured by analyzing ^{35}S-methionine-labeled lysates from infected tumor cells. In permissive tumor cell lines, such as HCT-116 and C33A, ONYX-015 infection resulted in the shutdown of host protein synthesis

as efficiently as wild-type virus (WtD), (Figure 1A). In contrast, the shutoff of host protein synthesis was impaired in ONYX-015-infected resistant tumor cell lines, such as U2OS and Ovcar-3.

ONYX-015-infected permissive tumor cells export late viral RNAs, such as fiber and hexon, five to ten times more efficiently than ONYX-015-infected primary cells (O'Shea et al., 2004). Therefore, we used an RNA fluorescent in situ hybridization assay (FISH) to visualize the localization of the late viral 100K mRNA in resistant and permissive tumor cell lines. In ONYX-015-infected U2OS cells, 100K mRNA export is delayed compared to WtD-infected cells (Figure 1B). In contrast, in HCT-116 cells, 100K mRNA is exported in ONYX-015 infection analogous to that of WtD (Figure 1C). These data suggest that differences in providing the late functions of E1B-55K underlie the variable ability of tumor cell lines to support ONYX-015 replication.

100K expression is rate limiting for ONYX-015 tumor cell replication

Several previous studies have shown that the presence of cytoplasmic fiber and hexon RNAs correlates with tumor cell permissivity to ONYX-015 (Goodrum and Ornelles, 1998; Goodrum and Ornelles, 1999; Harada and Berk, 1999). However, this does not appear to explain how host protein shutoff might also be achieved in the absence of E1B-55K. In Figure 1, we demonstrate that ONYX-015-infected resistant tumor cells fail to mediate host protein shutoff and inefficiently export 100K RNA compared to permissive cell lines. In adenovirus infection, 100K inhibits the translation of many cellular mRNAs but promotes the translation of late viral RNAs (Cuesta et al., 2000b; Hayes et al., 1990). Consequently, 100K mutant viruses are defective for host protein shutoff and replication (Hayes et al., 1990; Morin and Boulanger, 1986). Therefore, we hypothesized that the differences in host protein shutoff in ONYX-015-infected resistant versus permissive tumor cell lines may, in part, be explained by disparities in their expression levels of 100K.

Consistent with this hypothesis (and Figures 1B and 1C), 100K expression is defective in ONYX-015-infected U2OS cells but expressed at WtD levels in ONYX-015-infected HCT-116 cells (Figure 2A). We next investigated whether stable ectopic expression of 100K would render U2OS cells permissive for ONYX-015 replication. Ectopic 100K expression resulted in the enhanced expression of late viral proteins (such as hexon, penton, and fiber) in ONYX-015-infected U2OS cells (Figure 2B), consistent with 100K's role in promoting the translation of late viral RNAs. Moreover, siRNA-mediated knockdown of 100K reduced late viral protein expression in ONYX-015-infected HCT-116 cells (Figure 2C). In ONYX-015-infected U2OS cells, ectopic 100K also resulted in enhanced cytopathic effect (CPE) and a 10-fold increase in viral yield (Figures 2D and 2E). Nevertheless, ONYX-015 replication was not restored to WtD levels, possibly because 100K expression was still limiting or, more likely, due to the still defective export of other critical late viral RNAs. Notwithstanding, these data demonstrate that 100K expression is at least one of the critical rate-limiting factors for ONYX-015 replication and late protein production in resistant tumor cell lines.
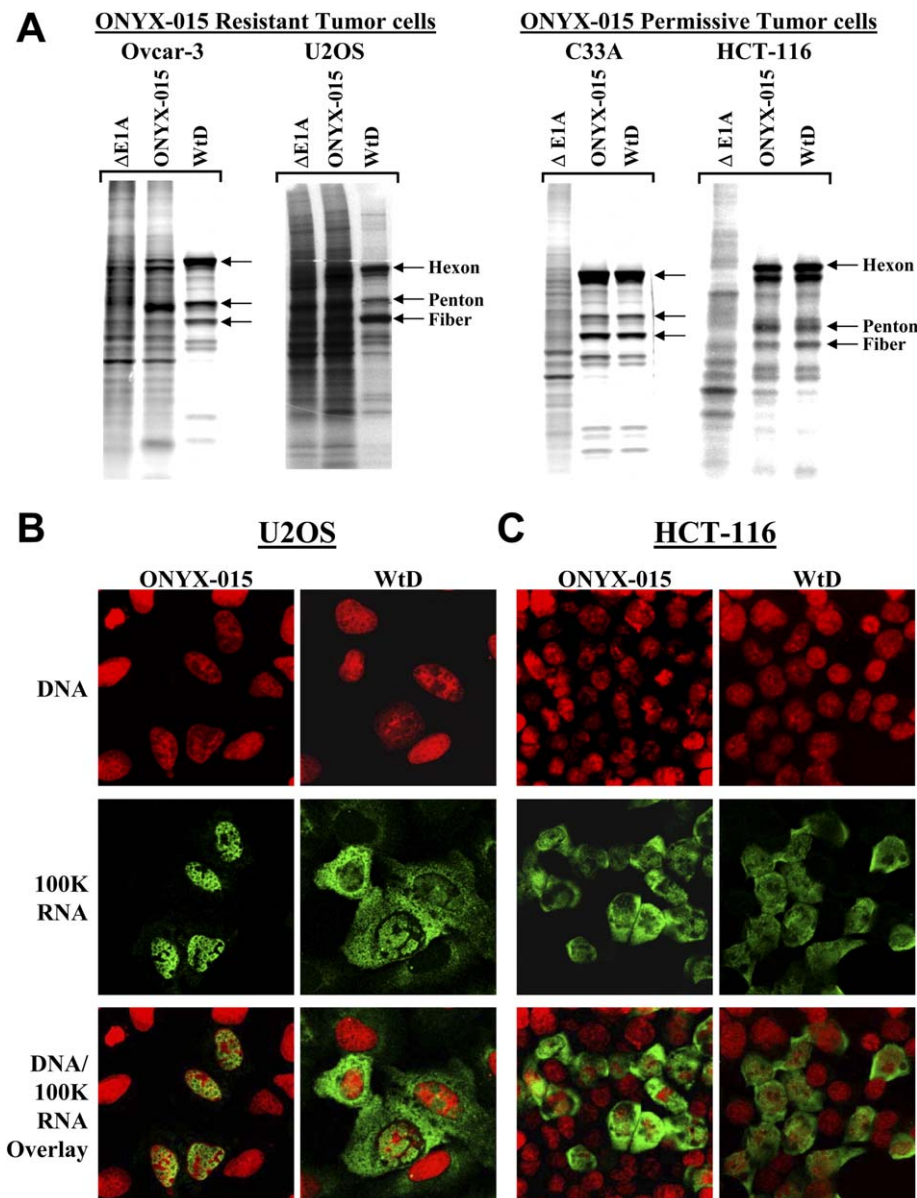
A

ONYX-015 Resistant Tumor cells
Ovcar-3   U2OS

ONYX-015 Permissive Tumor cells
C33A   HCT-116

← Hexon
← Penton
← Fiber

← Hexon
← Penton
← Fiber

B   U2OS

ONYX-015   WtD

DNA

100K RNA

DNA/ 100K RNA Overlay

C   HCT-116

ONYX-015   WtD

Figure 1. ONYX-015 replicates in tumor cells that provide the host protein shutoff and RNA export functions of E1B-55K

A: Host protein shutoff was measured in tumor cell lines infected with either ΔE1A (a nonreplicating virus and control for infection), ONYX-015, or wild-type virus (WtD). U2OS and Ovcar-3 minimally support ONYX-015 replication (resistant), while HCT-116 and C33A support ONYX-015 replication to WtD levels (permissive). Infected cells were pulsed with ^{35}S-methionine for 1 hr. Protein lysates were harvested, normalized, and separated on SDS-PAGE gels; exposed on a Storm phosphorimager; and analyzed using Molecular Dynamics software.

B and C: U2OS cells (**B**) and HCT-116 cells (**C**) were infected with either ONYX-015 or WtD and fixed at 24 hr postinfection (hpi). RNA fluorescent in situ hybridization (FISH) was performed using an antisense digoxigenin-labeled probe to 100K mRNA and detected with a FITC-labeled anti-digoxigenin antibody (green); nuclei are shown in red. Images were acquired on a Zeiss Meta confocal laser scanning microscope.

Heat shock rescues host protein shutoff and late viral protein expression in ONYX-015-infected U2OS cells

We conclude that the export and translation of late viral RNAs, such as 100K (this study), together with hexon and fiber (Goodrum and Ornelles, 1998; Goodrum and Ornelles, 1999; Harada and Berk, 1999), in the absence of E1B-55K, determine a tumor cell's ability to support ONYX-015 replication. Unfortunately, the mechanics and regulation of mammalian and adenoviral RNA export are poorly understood. In contrast, more is known about the control of mRNA translation, including that of late adenoviral RNAs. In Figure 2E, we show that ectopic 100K expression, which promotes late viral mRNA translation (Cuesta et al., 2000b), rescues ONYX-015 replication by up to 10-fold in the resistant U2OS tumor cell line. Therefore, we reasoned that selectively invoking 100K-like functions in resistant tumor

cells might significantly broaden the clinical application and activity of ONYX-015 as a cancer therapy.

In many ways, the cellular response to heat shock resembles the late stages of adenovirus infection. During a heat shock response, the translation of cellular mRNAs is inhibited, to prevent the further accumulation of misfolded proteins. However, heat shock mRNAs are preferentially translated via a ribosome shunting mechanism, whereupon they promote cell survival through their functions as molecular chaperones (Rhoads and Lamphear, 1995). The 5′UTR of mammalian *Hsp70* and late adenoviral mRNAs share significant structural homology, which facilitates their selective translation through a common mechanism in a cellular heat shock response or viral infection (Yueh and Schneider, 2000). *dl*1520/ONYX-015 has previously been described as a "cold-sensitive" virus and replicates poorly at low temperatures (Harada and Berk, 1999; Ho et al., 1982;
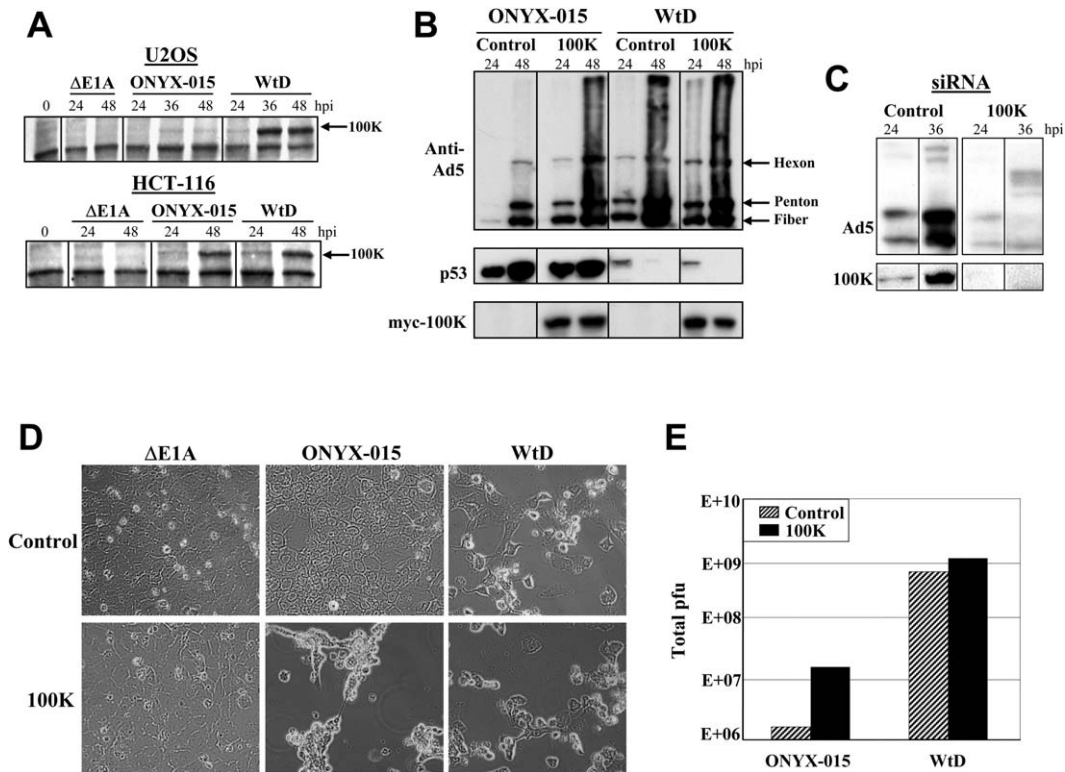
Figure 2. 100K expression is regulated by E1B-55K and rate limiting for ONYX-015 replication in tumor cells

A: 100K expression was analyzed by Western blotting of lysates from U2OS or HCT-116 tumor cells that had been infected with either ΔE1A, ONYX-015, or WtD.

B: Control and myc-100K-expressing U2OS cells were infected with either ONYX-015 or WtD. Protein lysates were examined by Western blotting for the expression of Ad5 proteins (hexon, penton, and fiber), p53, and myc-100K.

C: HCT-116 cells were transfected with either 100K siRNA or nonsilencing control siRNA and infected with ONYX-015. Protein lysates were examined for 100K and Ad5 late protein expression.

D and E: Stable U2OS cell clones for either myc-100K or control vector were infected with either ΔE1A, ONYX-015, or WtD. **D:** Cytopathic effect (CPE) was visualized at 72 hpi. **E:** ONYX-015 and WtD viral yield at 48 hpi was measured by quantifying total plaque-forming units (pfu) in 293E4s.

Leppard and Shenk, 1989). Therefore, we wondered if the "cold sensitivity" might instead be a "heat dependency," and if heat shock would enhance late viral protein expression in ONYX-015-infected U2OS cells.

We examined late protein expression in ONYX-015-infected U2OS cells at various temperatures. Temperatures of 39.4°C–40°C for 4–18 hr postinfection (hpi) were sufficient to enhance the expression of late viral proteins in ONYX-015-infected U2OS cells, although prolonged incubation at 44°C was too severe and inhibited late viral protein synthesis even in WtD-infected cells (Figure S1 and data not shown). Incubation at 39.4°C significantly rescues the expression of the majority of late viral proteins in ONYX-015-infected U2OS cells, including hexon, penton, fiber, V, VI, and VII (Figure 3A). We also observed a protein of approximately 33 kDa that is absent in ONYX-015-infected lysates compared to WtD, and which is not rescued by heat shock. The molecular weight of this protein would be consistent with that of the late viral L4-33K protein, which functions in virion assembly and the early to late switch in viral protein expression (Farley et al., 2004; Fessler and Young, 1999). However, 33K is not present in the mature virion, and antisera raised against mature viral particles would not be

expected to detect it. Nevertheless, given the functions of 33K, we are currently investigating the identity of this cryptic 33 kDa protein band.

Since 39.4°C is within the range of temperatures experienced by patients exposed to high doses of ONYX-015 (Reid et al., 2001), we chose to study the effect of this temperature on ONYX-015 replication further. Incubation at 39.4°C results in a bona fide cellular heat shock response as evidenced by the induction of heat shock mRNAs such as Hsp70, Crystallin αB, Hsp27, and Hsp90 (Figure 3B). In Figure 3C, we show that incubation at 39.4°C also rescued 100K expression in ONYX-015-infected U2OS cells and, consistent with this, host protein shutoff (Figure 3D).

Heat shock rescues the export of late viral RNAs in ONYX-015-infected U2OS cells

We next investigated the mechanism whereby heat shock rescues late viral protein synthesis and host protein shutoff in ONYX-015-infected U2OS cells. The inhibition of cap-dependent translation in mammalian cells exposed to chronic heat shock (44°C) is thought to be mediated through a reduction in soluble eIF-4G, and consequently phospho-eIF-4E, bound to
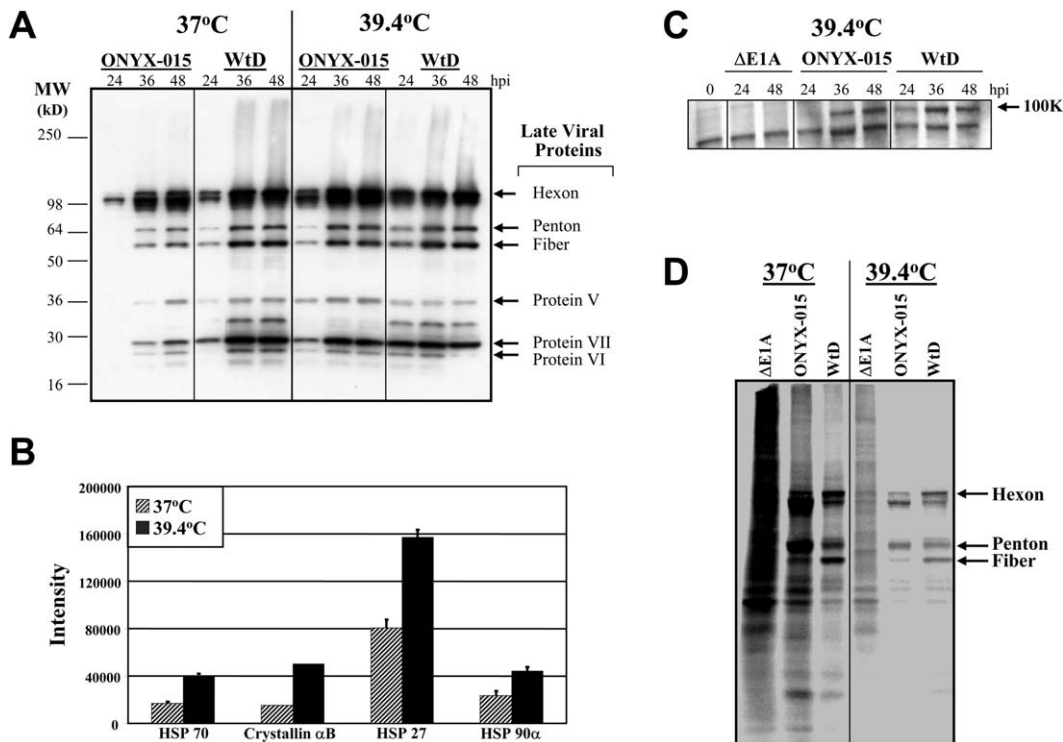
Figure 3. Heat shock rescues host protein shutoff and late viral protein production in ONYX-015-infected refractory tumor cells

A: U20S cells were infected with either ONYX-015 or WtD. Protein lysates were examined by Western blotting for the expression of late viral proteins. Molecular weight (MW) in kD and the identity of late viral proteins (based on known specificity of antisera and MW) are marked.

B: U2OS cells were incubated at either 37°C or 39.4°C for 24 hr. RNA was extracted and analyzed for the expression of Hsp genes using an Affymetrix HGU133A GeneChip. Intensity values were calculated using Affymetrix Microarray Suite software. The average values of three independent experiments are plotted.

C: U2OS cells were infected with either ΔE1A, ONYX-015, or WtD and incubated at 39.4°C. 100K expression was examined by Western blotting.

D: U2OS cells were infected with either ONYX-015 or WtD and incubated at 37°C or 39.4°C. Host protein shutoff was analyzed at 48 hpi.

methyl[7] GTP (Cuesta et al., 2000a). However, in Figure 4A, we show that incubation of U2OS cells at 39.4°C does not reduce either the total levels of phospho-eIF-4E or the fraction of eIF-4E capable of binding to the cap analog methyl[7] GTP sepharose. Indeed, this is consistent with the original report of heat shock effects on translation, which demonstrated that the eIF-4G-heat/stress granules do not form at lower temperatures (Cuesta et al., 2000a), such as, in our case, 39.4°C. Therefore, effects on eIF-4E at 39.4°C do not appear to account for the shutdown in cellular protein synthesis observed in ONYX-015-infected U2OS cells in Figure 3D.

We next investigated whether heat shock increased the transcription of late viral mRNAs such as 100K and fiber in ONYX-015-infected U2OS cells. However, the ratio of 100K or fiber mRNA levels in ONYX-015- versus WtD-infected U2OS cells did not increase at 39.4°C compared to 37°C (Figure 4B). In yeast, heat shock has been shown to induce the preferential nuclear export of Hsp mRNAs, while global RNA export is concomitantly inhibited (Saavedra et al., 1996; Saavedra et al., 1997). Therefore, we examined whether the incubation of ONYX-015-infected U2OS cells at 39.4°C rescues the export of late viral RNAs, which, as we have demonstrated, appears to be the major limitation to ONYX-015 replication in these cells. Figure 4C demonstrates that incubation at 39.4°C was

sufficient to rescue the export of 100K mRNA (and by inference host protein shutoff) in ONYX-015-infected U2OS cells. Heat shock also rescued, albeit to a lesser extent, the export of other late viral RNAs, such as fiber and hexon (Figure 4D and Supplemental Data, respectively). From this we conclude that incubation of ONYX-015-infected U2OS cells at 39.4°C phenocopies the functions of E1B-55K in the export of the late viral RNAs, 100K, hexon, and fiber, together with host protein shutoff.

Heat shock rescues ONYX-015 replication in nonpermissive tumor cells

We next determined whether heat shock-induced late viral RNA export was sufficient to rescue ONYX-015 replication in resistant tumor cells. Incubation of U2OS cells at 39.4°C resulted in CPE (Figure 5A) and a 30-fold increase in ONYX-015 viral yield (Figure 5B). In contrast, WtD yield increased by at most 2-fold at the elevated temperature, as has been noted before (Haviv et al., 2001).

We next investigated whether a heat shock response could rescue ONYX-015 replication in a panel of resistant tumor cell lines. Incubation of ONYX-015-infected MDA-MB-231, MDA-MB-453, SK-BR-3, HCC38, HCC3153, and MDA-MB-415 tumor cell lines at 39.4°C rescued the expression of late viral
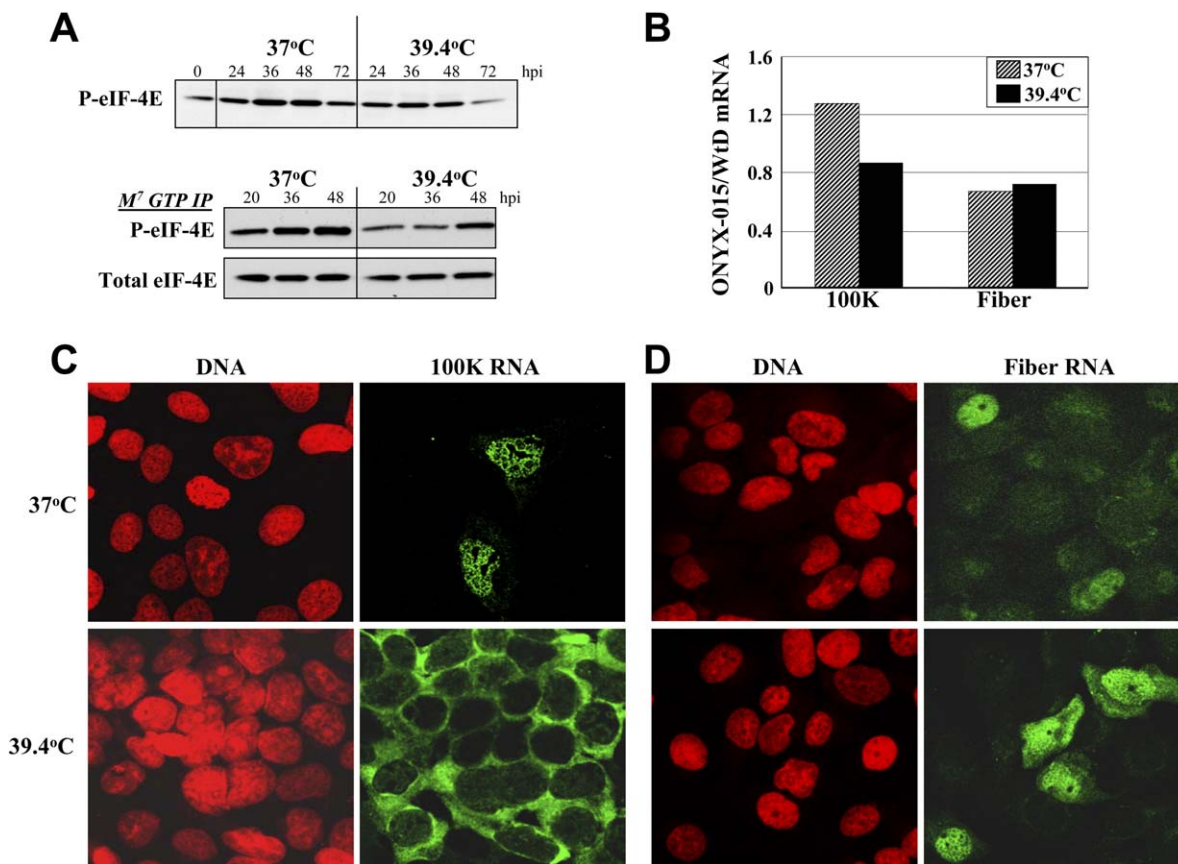
Figure 4. Heat shock rescues late viral RNA export in ONYX-015-infected U2OS cells

A: U2OS cells were infected with a ΔE1A virus and incubated at either 37°C or 39.4°C. Lysates were examined by Western blotting (upper panel) for phospho-ser-209-eIF-4E. In addition, the ability of both total and phospho-ser-209-eIF-4E to bind to capped mRNAs was examined by precipitation with the cap analog methyl^7 GTP sepharose (lower panel).

B: RNA was extracted from U2OS cells infected with either ONYX-015 or WtD and incubated at either 37°C or 39.4°C. Fiber and 100K mRNA levels were quantified by real-time Q-PCR and normalized relative to 18S.

C and D: U2OS cells were infected with ONYX-015 and incubated at either 37°C or 39.4°C. Cells were fixed at 24 hpi and analyzed for 100K (**C**) and fiber (**D**) RNA export by FISH.

proteins, as shown in Figure 5C. Moreover, incubation at 39.4°C also resulted in, on average, an 8-fold increase in ONYX-015 replication (Table 1). In contrast, the incubation of ONYX-015-infected permissive tumor cell lines, such as C33A, at 39.4°C resulted in at most a 2-fold increase in replication (Figure S3) and had minimal effects on late protein expression, ostensibly because E1B-55K late functions are already complemented.

Benzoquinoid ansamycins rescue ONYX-015 production in resistant tumor cells

The effects of elevated temperatures on ONYX-015 replication could be due to the destabilization of temperature-sensitive proteins and/or the induction of a cellular heat shock response. Therefore, we investigated whether pharmacological inducers of a heat shock response could also rescue ONYX-015 replication in resistant tumor cells. One such class of compounds is the benzoquinoid ansamycins, which include herbimycin A (Hegde et al., 1995), radicicol, geldanamycin, and 17-AAG (Neckers, 2002). These compounds block Hsp90 chaperone

function, thereby provoking a secondary misfolded protein/ heat shock response, which includes the induction of Hsp70. Herbimycin A resulted in a strong induction of Hsp70 in ΔE1A (a nonreplicating virus control for infection)-infected U2OS cells, consistent with a bona fide heat shock response (Figure 6A). Herbimycin A, like elevated temperature, also rescued 100K protein expression (Figure 6B) and host protein shutoff in ONYX-015-infected U2OS cells (Figure 6C). In addition, herbimycin A increased ONYX-015 viral yield, without overtly affecting that of WtD, in infected U2OS cells (Figure 6D). We also conducted a dose-response experiment with herbimycin A/geldanamycin to determine if there was an optimal concentration that would maximally rescue ONYX-015 replication in U2OS cells (Figure 6E). We found that a concentration of 200–400 ng/ ml geldanamycin increased ONYX-015 viral yield up to 130-fold, close to a complete rescue to WtD levels.

Similar to incubation at 39.4°C, treatment with benzoquinoid ansamycins enhances the yield of ONYX-015 in a panel of resistant tumor cell lines (Table 1). Geldanamycin or 17-AAG resulted in a 10-fold, or greater, increase in ONYX-015 replication
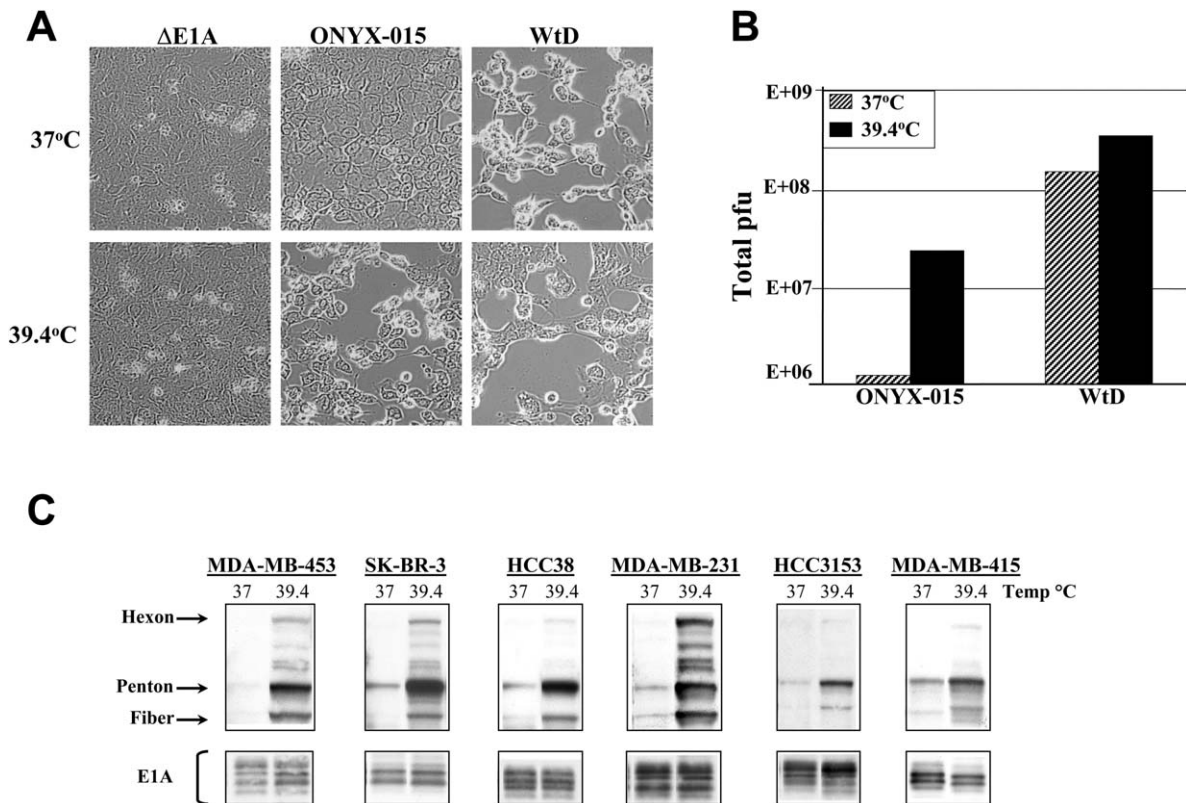
Figure 5. Heat shock rescues ONYX-015 replication in resistant tumor cells

A: CPE at 64 hpi in U2OS cells infected with either ΔE1A, ONYX-015, or WtD and incubated at either 37°C or 39.4°C.

B: U2OS cells were infected with either ONYX-015 or WtD and incubated at either 37°C or 39.4°C. Virus replication was measured at 48 hpi.

C: Resistant tumor cells were infected with ONYX-015 and incubated at either 37°C or 39.4°C. Protein lysates were harvested at 24 hpi and examined by Western blotting for the expression of E1A and late viral proteins.

in eight out of ten resistant tumor cell lines tested. Taken together, these data demonstrate that drugs that elicit a heat shock response can significantly rescue ONYX-015 replication in many refractory tumor cell lines.

Induction of a cellular heat shock response fails to rescue ONYX-015 replication in primary human cells

To have a potential clinical application, the induction of a cellular heat shock would have to maintain, or indeed improve, the therapeutic index of ONYX-015 for tumor versus normal cells. Previously, we found that ONYX-053, a novel adenovirus mutant that is defective for E1B-55K-mediated p53 degradation but retains E1B-55K late functions (Shen et al., 2001), replicated to almost wild-type virus levels in primary cells (O'Shea et al., 2004). Thus, our attempts at genetically engineering an E1B-55K mutant virus with enhanced replication in resistant tumor cell lines also ablated oncolytic selectivity. Therefore, we would have expected the induction of a cellular heat shock response to have a similar effect, and to rescue ONYX-015 replication in primary cells, obviating any potential clinical utility of a heat shock/ONYX-015 combination cancer therapy.

Notwithstanding, we examined the effect of elevated temperature and herbimycin A on ONYX-015 replication in primary quiescent small airway epithelial cells (SAECs). We used quies-

cent primary cells, to try to reflect the physiological state of the majority of normal epithelial cells within the body. In contrast to resistant tumor cells, heat shock did not rescue 100K expression in ONYX-015-infected primary SAECs (Figure 7A). Indeed, in infected SAECs, ONYX-015 replication was further attenuated by incubation at 39.4°C, or by treatment with herbimycin A, as shown in Figure 7B. Surprisingly, WtD replication was also attenuated by up to 50-fold under these conditions. Moreover, these conclusions hold true for a panel of primary cells. Incubation at 39.4°C or treatment with herbimycin A/geldanamycin, for the most part, decreased ONYX-015 replication in primary keratinocytes, astrocytes, mammary epithelial cells, and bronchial epithelial cells (Figure 7C and Table S2). Treatment with 17-AAG resulted in up to 2.1-fold increases in ONYX-015 viral yield in primary cells, but in comparison to tumor cells, these effects are minimal. In addition, heat shock appears to decrease both WtD and ONYX-015 viral yield irrespective of whether normal cells were proliferating or rendered quiescent prior to infection.

We examined whether a heat shock response resulted in premature apoptosis, cell cycle arrest, or defective p53 degradation in adenovirus-infected primary cells. Heat shock had no appreciable effect on apoptosis or cell cycle entry, as evidenced by PARP/caspase 3 cleavage or cyclin A induction (an

Table 1. ONYX-015 replication in resistant tumor cells incubated at either 37°C or 39.4°C, or in the presence of benzoquinoid ansamycins

Cell line	Treatment	Viral yield (pfu × 10⁶)	Fold change relative to 37°C
U2OS	37°C	1.05	1.0
(48 hpi)	39.4°C	13.52	12.9
(72 hpi)	37°C	1.86	1.0
	herbimycin A	29.67	15.9
(72 hpi)	37°C	2.49	1.0
	geldanamycin	323.11	129.6
	radicicol	83.59	33.5
MDA-MB-231	37°C	8.67	1.0
(72 hpi)	geldanamycin	122.75	14.2
	17-AAG	288.38	33.2
	39.4°C	162.86	18.8
MDA-MB-453	37°C	68.08	1.0
(48 hpi)	geldanamycin	123.24	1.8
	17-AAG	361.40	5.3
	39.4°C	315.56	4.6
Ovcar-3	37°C	9.59	1.0
(72 hpi)	geldanamycin	98.42	10.3
	herbimycin A	45.85	4.8
	39.4°C	29.52	3.1
SK-BR-3	37°C	18.21	1.0
(72 hpi)	geldanamycin	190.72	10.5
	17-AAG	44.08	2.4
	39.4°C	99.36	5.5
MDA-MB-415	37°C	3.49	1.0
(48 hpi)	geldanamycin	34.68	9.9
	39.4°C	83.74	24.0
MDA-MB-436	37°C	8.97	1.0
(48 hpi)	geldanamycin	89.38	10.0
	39.4°C	33.25	3.7
LNCaP	37°C	BLD (<10⁶)	1.0
(72 hpi)	geldanamycin	6.38	>6.3
	herbimycin A	6.00	>6.0
	39.4°C	BLD (<10⁶)	
HCC38	37°C	7.80	1.0
(48 hpi)	geldanamycin	14.20	1.8
	17-AAG	4.16	0.5
	39.4°C	21.51	2.8
HCC3153	37°C	13.53	1.0
(72 hpi)	geldanamycin	25.83	1.9
	17-AAG	17.18	1.3
	39.4°C	24.22	1.8

Resistant tumor cell lines were infected with ONYX-015 and incubated at either 37°C or 39.4°C, or in the presence of benzoquinoid ansamycins. Radicicol was used at 5 μM; 17-AAG was used at 100 ng/ml; herbimycin A was used at 500 ng/ml, except for LNCaP, which received 2500 ng/ml; and geldanamycin was used at 100 ng/ml, except for U2OS, Ovcar-3, and LNCaP, which received 400 ng/ml, and MDA-MB-231 and -453, which received 500 ng/ml. Viral yield was measured at 48 and 72 hpi by quantifying total plaque-forming units (pfu) in 293E4s. Data for either 48 or 72 hpi are shown and reflect the time point at which viral yield (untreated) peaked in the respective tumor cell lines. BLD, below limit of detection; hpi, hours postinfection.

E2F target), respectively (Figure 7D). WtD degraded p53 at both 37°C and 39.4°C, implying that the effects of heat shock in decreasing wild-type viral replication were not related to effects on p53. This suggests that the disparity between normal and tumor cells with respect to the effect of heat shock on virus production may be unrelated to the disruption of the RB/p53 checkpoints and reflect a novel difference. Taken together, these data indicate that the combined use of ONYX-015 with agents that elicit a heat shock response could significantly improve and broaden ONYX-015's therapeutic utility.

Discussion

Oncolytic viruses are a novel and promising approach to cancer therapy. ONYX-015 was one of the first oncolytic adenoviral agents to be tested in the clinic, where it was found to be safe with evidence of promising activity in Phase I and II clinical trials (Khuri et al., 2000; Kirn, 2001; Reid et al., 2002; Ries and Korn, 2002; Rudin et al., 2003). Nevertheless, ONYX-015 has yet to be evaluated in Phase III clinical trials and, therefore, remains an unproven therapy. Two of the major factors that stalled the further clinical evaluation of ONYX-015 were the controversy surrounding its p53 tumor selectivity, together with its variable tumor cell replication. Thus, while many cancer patients exhibited sustained complete/partial responses to ONYX-015 therapy, which would potentially recommend its clinical approval, many were resistant to ONYX-015, for reasons apparently unrelated to the p53 status of their tumors (Khuri et al., 2000). Recently, the role of p53 in determining ONYX-015 tumor selectivity has been resolved. In ONYX-015-infected cells, p53 is induced, but not activated, and therefore plays a minimal role in limiting viral replication, at least in vitro (Hobom and Dobbelstein, 2004; O'Shea et al., 2004). Instead, the complementation of E1B-55K late functions in tumor cells appears to be the major determinant of ONYX-015 oncolytic selectivity (Goodrum and Ornelles, 1998; Rothmann et al., 1998; Harada and Berk, 1999; Turnell et al., 1999; O'Shea et al., 2004).

The aim of this study was to determine the molecular mechanism underlying the variable replication of ONYX-015 in tumor cells with the ultimate goal of sensitizing refractory tumor cells to ONYX-015 therapy. Here, we demonstrate that a tumor cell's ability to provide the host protein shutoff and late viral RNA export functions of E1B-55K determines ONYX-015 permissivity/resistance. Nevertheless, it was not clear how altered RNA export in permissive tumor cells could provoke host protein shutoff, or if indeed this was a distinct property of permissive tumor cell lines. Here, we demonstrate that E1B-55K regulates the export of 100K RNA, which, upon translation in the cytoplasm, functions downstream of E1B-55K to mediate the shutdown of host protein synthesis and enhance the expression of late viral proteins. In addition, 100K also plays an important role in hexon trimerization and viral assembly (Cepko and Sharp, 1982; Morin and Boulanger, 1986). We show that 100K expression is limiting in ONYX-015-infected resistant tumor cells, and necessary in ONYX-015-infected permissive tumor cells. These data resolve the dual functions of E1B-55K in host protein shutoff and late viral RNA export to a single function: late viral RNA export of capsid RNAs such as fiber and hexon, and 100K RNA for host protein shutoff and viral morphogenesis. Similarly, the ability of permissive tumor cells to support ONYX-015 replication also resolves to a single property: the export of late viral RNAs in the absence of E1B-55K.

Our next objective was to try to extend ONYX-015 replication to refractory tumor cells. However, the molecular mechanisms underlying both adenoviral and mammalian RNA export are poorly understood, and not obviously tractable to manipulation. Therefore, given that ectopic expression of 100K in U2OS cells increased ONYX-015 replication by up to 10-fold, we initially chose to focus on strategies that might be used therapeutically to mimic 100K-mediated translation of late viral RNAs. However, neither rapamycin treatment, which inhibits mTOR, nor SB203580 together with U0126 treatment, which
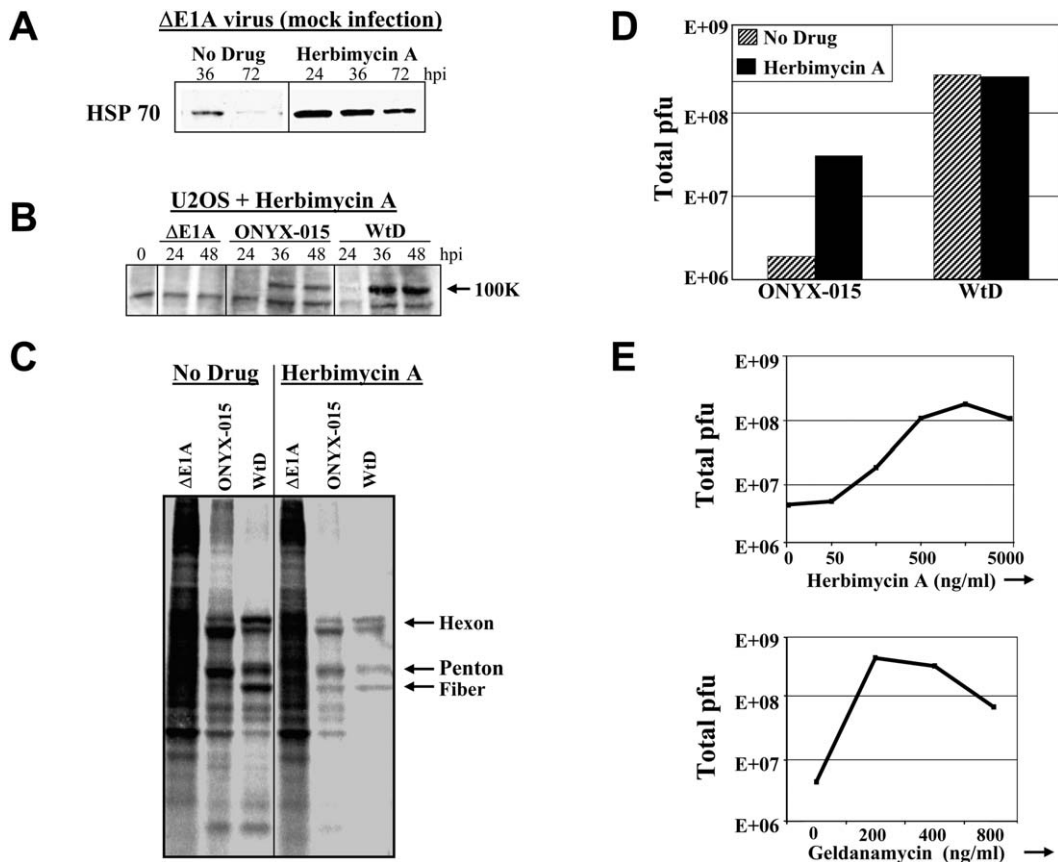
Figure 6. Herbimycin A and geldanamycin rescue ONYX-015 in resistant U2OS cells

A: U2OS cells were mock infected with ΔE1A virus and treated with herbimycin A (500 ng/ml). Hsp70 expression was analyzed by Western blotting.

B: U2OS cells were infected with either ΔE1A, ONYX-015, or WtD and treated with herbimycin A (500 ng/ml). Protein lysates were analyzed for 100K expression by Western blotting.

C: Host protein shutoff was examined at 48 hpi in U2OS cells infected with either ΔE1A, ONYX-015, or WtD and incubated in the presence/absence of herbimycin A (500 ng/ml).

D: ONYX-015 or WtD viral yield was measured in U2OS cells incubated in the presence/absence of herbimycin A (500 ng/ml).

E: A dose-response curve with herbimycin A or geldanamycin was performed in ONYX-015-infected U2OS cells. Viral yield was measured at 48 hpi.

inhibits mnk-1 phosphorylation of eIF-4E (Wang et al., 1998), nor expression of the poliovirus 2a^{pro}, which cleaves eIF-4G (Castrillo and Carrasco, 1987), rescued 100K functions and late viral protein production in ONYX-015-infected U2OS cells (data not shown). Indeed, this is perhaps not surprising in light of recent elegant studies, which revealed that 100K possesses a selective binding element for the tripartite leader of late viral RNAs, and simultaneously binds to eIF-4G (consequently displacing mnk), to drive 40S ribosome recruitment and the translation of viral RNAs (Cuesta et al., 2004). Nevertheless, our inhibitor experiments imply that any nominal strategy that decreases the translation of cellular mRNAs does not necessarily mimic the specific effects of a heat shock response on ONYX-015 replication.

E1B-defective adenoviruses have been described as "cold-sensitive viruses" (Ho et al., 1982). Recent studies, which made use of temperature-sensitive p53 mutants (stable at 32°C and unstable at 39°C) to examine the role of p53 on ONYX-015 replication, also reported a confounding effect of temperature on ONYX-015 replication, irrespective of whether p53 was pre-

sent or not. Indeed, a severe defect in late viral protein synthesis was observed at 32°C in the H1299 tumor cell line (Harada and Berk, 1999). However, there was only a modest, at most 2-fold, decrease in cytoplasmic fiber and hexon mRNA, and so effects of temperature on viral protein translation were conjectured as the likely cause. These studies, together with the knowledge that heat shock and late viral RNAs have structurally similar 5′UTRs (Yueh and Schneider, 2000), prompted us to determine if heat shock could rescue ONYX-015 replication in resistant tumor cells. Incubation of refractory tumor cells at 39.4°C rescued late viral protein expression and host protein shutoff. The relatively mild heat shock temperature of 39.4°C did not affect phospho-eIF-4E binding to methyl^7 GTP in ONYX-015-infected U2OS cells. Instead, heat shock induces resistant tumor cells to export late viral RNAs, the primary impediment to ONYX-015 replication in these cells. Thus, heat shock-mediated host protein shutoff in ONYX-015-infected nonpermissive tumor cells is most likely a secondary result of 100K RNA export and expression.

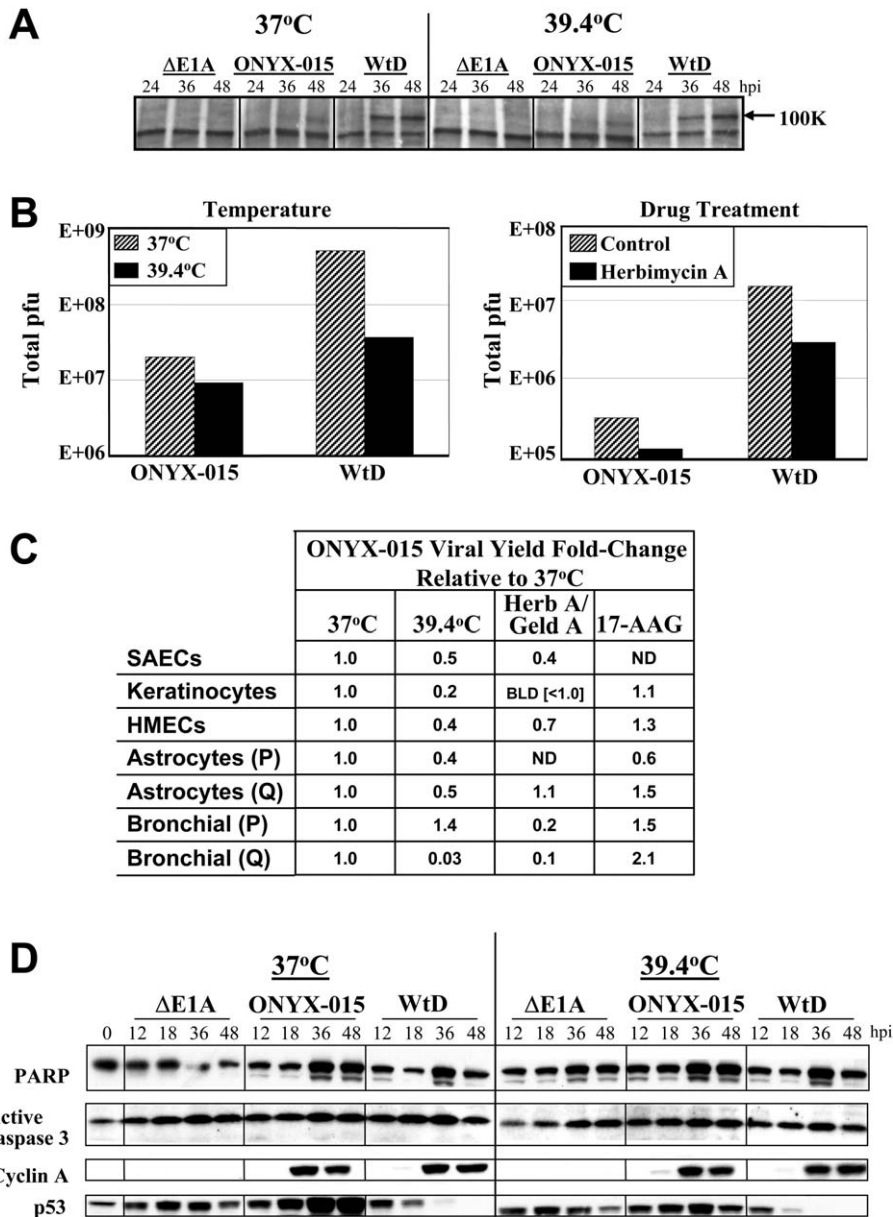We find that late viral messages depend on E1B-55K for their

A

B

C

	ONYX-015 Viral Yield Fold-Change Relative to 37°C			
	37°C	39.4°C	Herb A/ Geld A	17-AAG
SAECs	1.0	0.5	0.4	ND
Keratinocytes	1.0	0.2	BLD [<1.0]	1.1
HMECs	1.0	0.4	0.7	1.3
Astrocytes (P)	1.0	0.4	ND	0.6
Astrocytes (Q)	1.0	0.5	1.1	1.5
Bronchial (P)	1.0	1.4	0.2	1.5
Bronchial (Q)	1.0	0.03	0.1	2.1

D

Figure 7. Induction of a cellular heat shock response fails to rescue ONYX-015 replication in primary human cells

A: Primary quiescent small airway epithelial cells (SAECs) were infected with either ΔE1A, ONYX-015, or WtD and incubated at either 37°C or 39.4°C. 100K expression was examined by Western blotting.

B: Viral production was measured in SAECs that had been infected with either ONYX-015 or WtD and incubated at either 37°C or 39.4°C, or in the presence of 500 ng/ml herbimycin A.

C: The ratio/fold change of ONYX-015 viral yield at 72 hpi in infected quiescent (Q) or proliferating (P) primary cells incubated at either 39.4°C, or in the presence of either herbimycin A (SAECs), geldanamycin (500 ng/ml), or 17-AAG (100 ng/ml), is shown relative to cells incubated at 37°C. Actual titers obtained in these experiments are included in Table S2. Herb A, herbimycin A; Geld A, geldanamycin; HMECs, human mammary epithelial cells; BLD, below limit of detection; ND, not done.

D: SAECs were infected with either ΔE1A, ONYX-015, or WtD and incubated at either 37°C or 39.4°C. Protein lysates were harvested and examined by Western blotting with the indicated antibodies.

export to different degrees, with hexon > fiber > 100K, and that this is also reflected in the extent to which a heat shock response rescues their export (Figures 4C and 4D and Figure S2). This may reflect the temporal expression of these messages (Farley et al., 2004), their distance from the late viral promoter (Flint and Gonzalez, 2003), or molecular motifs within these RNAs that influence their mode of export. In addition, the extent to which late viral proteins depend on E1B-55K for their expression is not absolute but instead particularly exacerbated early in infection. One explanation for this is that late viral RNA transcripts accumulate over time and so can more effectively compete with cellular RNAs for nuclear export and/ or translation, despite the absence of E1B-55K/100K. In addition, 100K and L4-33K have been proposed to contribute to a "feed-forward" activation mechanism in mediating the early/ late switch (Farley et al., 2004), that may also account for the

kinetics of late protein expression observed in ONYX-015-infected cells. Nevertheless, the ability of heat shock to rescue late viral RNA export and consequently ONYX-015 replication indicates that it is a critical rate-limiting factor in resistant tumor cells.

We also demonstrate that pharmacological inducers of a heat shock response, such as the benzoquinoid ansamycins, rescue late protein expression, host shutoff, and viral replication in ONYX-015-infected resistant tumor cells. These agents inhibit Hsp90, which triggers a misfolded protein response and induction of Hsp70, akin to elevated temperature (Hegde et al., 1995). Therefore, the effects of heat shock on late viral RNA export are unlikely to be mediated through Hsp90, given that herbimycin A inhibits this family of Hsps. Recently, the induction of Hsp70 and Hsp40 by the avian adenovirus protein GAM-1 was shown to be essential for virus replication, and

GAM-1 mutants were partially rescued by heat shock (Glotzer et al., 2000). However, Hsp70 is unlikely to mediate the effects of heat shock in ONYX-015 replication, as it is potently induced by E1A (the GAM-1 functional equivalent) in both wild-type (Nevins, 1982; Wu et al., 1986) and ONYX-015-infected cells (data not shown). Incubation of resistant tumor cells at 39.4°C for 10 hr early in infection is sufficient to rescue the effects of E1B-55K in late viral replication. This is consistent with the induction of an ordered sequelae of events during a heat shock response, such as activation of the heat shock transcription factor HSF-1 by both temperature (Cotto and Morimoto, 1999) and geldanamycin (Zou et al., 1998). However, overexpression of a constitutively active HSF-1 (Hegde et al., 1995) fails to rescue late viral protein expression in ONYX-015-infected U2OS cells (data not shown). The preferential export of heat shock RNAs has previously been observed upon heat shock of yeast, but the molecular mechanism is controversial, and the mammalian factors remain to be elucidated (Jensen et al., 2001; Saavedra et al., 1996; Santoro, 2000; Vainberg et al., 2000). Therefore, a novel component of the heat shock pathway appears to mediate these effects, which we are currently investigating.

Our data suggest that heat shock and late viral RNAs may impinge on a common molecular mechanism for their nuclear export. Consistent with this, *Hsp70* mRNA continues to be exported to the cytoplasm late in viral replication, when the transport of most cellular RNAs is inhibited (Moore et al., 1987). From these data, we infer that late viral and *Hsp* RNAs may share a molecular resemblance that dictates their mode of export. The 5′UTR of late adenoviral RNAs has structural homology to the 5′UTR of *Hsp70* and 18S rRNA (Yueh and Schneider, 2000), which underlies their common property to be translated by ribosome shunting. In addition to its role in translation, the 5′UTR of late viral mRNAs has also been shown to play a role in export (Huang and Flint, 1998). Taken together, these data suggest that heat shock and late viral RNAs may share a common strategy for both translation and nuclear export that is specified by their 5′UTRs.

The upregulation of Hsps in tumor cells is well documented (Jolly and Morimoto, 2000). Therefore, it is intriguing to speculate that permissive tumor cells may constitutively deregulate cellular players in the RNA export pathway that play a role in heat shock/stress responses. Interestingly, the localization of YB-1, which plays a role in the transcription of multidrug resistance genes, is affected by both E1B-55K and heat shock (Holm et al., 2002; Holm et al., 2004; Raffetseder et al., 2003) and has recently been shown to have RNA binding functions that regulate mRNA translation (Bader et al., 2003).

These data have important and immediate clinical implications for ONYX-015 cancer therapy. Incubation at 39.4°C, or treatment with geldanamycin/17-AAG, enhances the replication of ONYX-015 in a panel of resistant tumor cell lines. Indeed, geldanamycin/17-AAG resulted in a 10-fold, or greater, increase in ONYX-015 viral yield in eight out of ten resistant tumor cell lines tested. Nevertheless, inevitably, as with any therapy, in some tumor cell lines, such as HCC38 and HCC3153, geldanamycin resulted in only a 2- to 3-fold increase in ONYX-015 replication. Reasons for this may include a common polymorphism in DT-diaphorase (Kelland et al., 1999), or differences in the expression of the latter together with CYP3A4 (Egorin et al., 1998), quinine metabolizing en-zymes that are thought to affect the efficacy of geldanamycin/17-AAG. Also, while we performed dose-response experiments in U2OS cells to determine an optimal concentration of geldanamycin that rescued ONYX-015 replication by up to 130-fold (almost to the level of wild-type virus) (Figure 6E), due to practical considerations, this was not possible for all tumor cell lines tested. The ability of heat shock-induced late viral RNA export to fully rescue ONYX-015 to wild-type virus levels may also be compromised by "off-target" effects that negatively impact viral replication. Nevertheless, our data indicate that the induction of a cellular heat shock could extend ONYX-015 oncolytic therapy to many formerly refractory tumor cells.

The effects of heat shock in enhancing ONYX-015 replication are restricted to tumor cells and, surprisingly, impair both wild-type and ONYX-015 viral replication in primary SAECs. This may reflect a differential heat shock response in tumor versus normal cells. Indeed, tumor-specific differences in Hsp regulation (Kamal et al., 2003) have been proposed to underlie the cancer selectivity of drugs, such as geldanamycin/17-AAG, which are currently undergoing clinical evaluation. Notwithstanding, our data indicate that heat shock appreciably enhances the therapeutic index of ONYX-015 for tumor versus normal cells. This is in sharp contrast to results obtained with ONYX-053 (Shen et al., 2001), a mutant virus that retains E1B-55K late functions to replicate more efficiently in resistant tumor cells but also replicates to almost wild-type virus levels in primary cells (O'Shea et al., 2004). Moreover, patients treated with high doses of ONYX-015 in clinical trials have experienced elevated body temperatures, presumably through inflammatory effects of virus infusion (Reid et al., 2001). Indeed, these effects have recently been proposed as a factor that may have significantly affected the clinical outcome of ONYX-015 therapy (Thorne et al., 2005). Our data suggest that a clinical strategy that does not advocate the use of pharmacological agents to suppress fever would favor the tumor-selective replication of ONYX-015. Finally, this study indicates that induction of a heat shock response by pharmacological agents (that could potentially be administered systemically) or local hyperthermia, could greatly augment and broaden ONYX-015's clinical utility as a cancer therapy.

Experimental procedures

Cell culture
Primary cells were obtained from BioWhittaker, cultured as per the manufacturer's recommendations, and rendered quiescent through contact inhibition followed by prolonged (14–18 days) incubation in complete medium. Tumor cells were cultured in DMEM, except HCT-116 (McCoy's) and HCC3153, HCC38, Ovcar-3 and LNCaP, (RPMI-1640), all of which were supplemented with 10% fetal calf serum. U2OS 100K and control stable cell clones were established by transfection with pcDNA3-myc-100K or control vector, respectively, followed by selection in 500 μg/ml G418. Benzoquinoid ansamycins were obtained from Sigma.

Viral infections and quantification
Viruses included ΔE1A, an E1A-deleted adenovirus (*dl*312), wild-type virus, WtD, and E1B-55K-deleted *dl*1520/ONYX-015 (Barker and Berk, 1987). Viral infections and viral replication/yield assays were carried out as described previously (Johnson et al., 2002; O'Shea et al., 2004), at multiplicity of infections (MOI) that had been determined experimentally (Supplemental Data).

Western blotting and protein translation assays
Cell lysates and immunoblotting were carried out as described previously (O'Shea et al., 2004). For primary antibodies, see the Supplemental Data.

Host protein shutoff assays were performed by analyzing normalized protein lysates from ^{35}S-methionine-labeled cells (Translabel, NEN) as described previously (O'Shea et al., 2004). For methyl[7] GTP assays, protein lysates were prepared in 0.5% NP-40 buffer and precipitated with methyl[7] GTP sepharose as described previously (O'Shea et al., 2005).

siRNA
100K siRNA smart pool was obtained from Dharmacon. HCT-116 cells were transfected with either 100 μM 100K silencing siRNA or nonsilencing control siRNA, using Lipofectamine Plus (Invitrogen). Four hours posttransfection, media were replaced and cells were infected with adenovirus.

RNA FISH
RNA FISH protocols and fiber/hexon probes were as described previously (O'Shea et al., 2004). For 100K FISH probe primers, see the Supplemental Data. Sense and antisense RNA probes were synthesized by in vitro transcription with either T7 or T3 RNA polymerase, respectively, using the Boehringer DIG RNA labeling kit. DNA was counterstained with either TO-PRO-3 or DAPI (Molecular Probes), and cells were analyzed on a Zeiss Meta Laser scanning confocal microscope.

Real-time Q-PCR analysis
RNA was isolated and reverse transcribed, and quantitative PCR analysis was performed on an ABI Prism 7700, as described previously (O'Shea et al., 2004). All measurements were performed in triplicate. For normalization, cDNA equivalent to 12.5 ng input RNA was measured in triplicate for ribosomal 18S transcripts (PDAR 18S, ABI). Taqman primers/probes for 100K and fiber were as described previously (O'Shea et al., 2004).

Hsp expression profiling
RNA was extracted using Trizol (Gibco) and Qiagen RNeasy. Total RNA (20 μg) was used to prepare biotin-labeled cRNA according to Affymetrix protocols. RNA/DNA quality/quantity was determined on an Agilent Bioanalyser 2100. cRNA was hybridized to Affymetrix HG U133A chips at the UCSF Gladstone Genomics Core using standard protocols. Data were analyzed using Affymetrix MAS 5.1 software and normalized using Robust Multichip Analysis algorithms (Irizarry et al., 2003). Experiments were performed in triplicate.

Supplemental data
The Supplemental Data include Experimental Procedures, three figures, and two tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/8/1/61/DC1/.

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