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Viral IL-6–Induced Cell Proliferation and Immune Evasion of Interferon Activity

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Lymphoma cells infected with Kaposi's sarcoma–associated herpesvirus are autocrine dependent on virus-derived interleukin-6 (IL-6), but not on cellular IL-6. During viral infection, host cells induce the antiviral factor interferon (IFN) to up-regulate p21, initiate cell cycle arrest, and inhibit virus replication. Viral IL-6, however, blocks IFN signaling. A viral transcriptional program exists in which only the viral IL-6 gene is directly activated by IFN-α, allowing the virus to modify its cellular environment by sensing and responding to levels of intracellular IFN signaling. The human cytokine cannot mimic this effect because IFN-α down-regulates the IL-6 receptor, gp80. Viral IL-6 bypasses the gp80 regulatory checkpoint by binding directly to the gp130 transducer molecule, resulting in tumor cell autocrine dependence on the viral cytokine for proliferation and survival.

Viral inhibition of host defenses has been linked to the proliferative properties of some virus-infected tumors, because of the overlapping nature of immune and tumor-suppressor signaling pathways (*1*). Kaposi's sarcoma herpesvirus (KSHV) is a non-integrated, episomal DNA virus possessing a virus-derived cytokine, vIL-6, that is expressed in infected primary effusion lymphoma (PEL) cells (*2–4*). These cells become autocrine dependent on vIL-6 but not on the human cell–derived cytokine hIL-6 (*5*), a B cell proliferation factor. In the absence of vIL-6 or when vIL-6 signaling is blocked, these autocrine-dependent cells stop dividing and undergo apoptosis. vIL-6 induces B cell proliferation and contributes to in vitro cell transformation, and thus may play a critical role in KSHV-related hematopoietic tumors such as PEL and multicentric Castleman's disease (CD) (*6–8*). It probably does not appreciably contribute to Kaposi's sarcoma (KS), in which alternative viral transcription programs are operative, because vIL-6 is variably expressed in this endothelial cell tumor.

The reasons why a human cell would become dependent on an exogenous, virus-derived, IL-6–like cytokine are puzzling. Despite intensive study, no major differences in downstream signaling have been found for vIL-6 and hIL-6 (*9*, *10*). The viral and human cytokines, however, differ in their receptor interactions. hIL-6 binds to a specific receptor, gp80, which forms a complex with the transmembrane gp130 transducer molecule responsible for carrying the IL-6 signal across plasma membranes (*11*). Unlike hIL-6, vIL-6 directly engages gp130, but once gp130 is activated, downstream signaling for the two cytokines is similar (*12–14*).

We hypothesized that KSHV-infected cells would become autocrine dependent on vIL-6 if the viral cytokine protects cells against innate immune defenses triggered by viral infection. Interferons (IFNs) are cytokines induced during viral infection to generate an antiviral cellular state and can initiate cell type–dependent growth arrest and apoptosis (*15*, *16*). Under low-serum conditions,

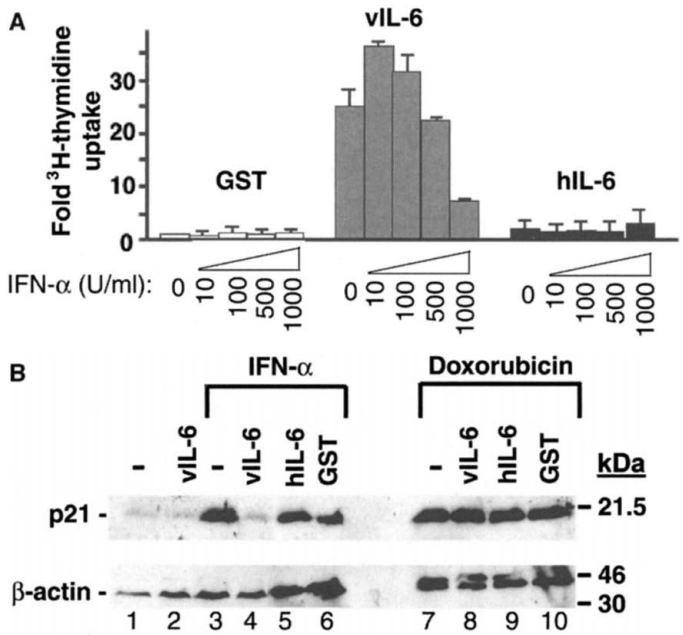

Fig. 1. vIL-6 inhibits the cytostatic effects of IFN-α on KSHV-infected PEL cells. **(A)** vIL-6 but not hIL-6 allows BCP-1 cell proliferation ([^3H]thymidine uptake) in low-serum media in the presence of IFN-α. Recombinant vIL-6, hIL-6, or GST was added to culture media at 100 ng/ml with cells harvested after 48 hours. **(B)** vIL-6 inhibits IFN-α–dependent but not p53-dependent up-regulation of the $p21^{CIP1/WAF1}$ cyclin-dependent kinase inhibitor. $p21^{CIP1/WAF1}$ immunoblotting was performed on BCP-1 cells after 16 hours of vIL-6, hIL-6, or GST treatment (100 ng/ml each) together with 500 IU of IFN-α or 0.4 M doxorubicin. IFN-α induces $p21^{CIP1/WAF1}$ protein expression (lane 3) that is antagonized by vIL-6 (lane 4) but not by hIL-6 (lane 5) or GST (lane 6). $p21^{CIP1/WAF1}$ protein induced by 0.4 M doxorubicin is unaffected on addition of exogenous cytokines or GST (lanes 7 to 10). β-actin is shown for loading comparison.

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vIL-6 initiated cell cycle entry and prevented cell death of BCP-1 cells (a B cell line infected with KSHV alone). This effect was partially antagonized by IFN-α (Fig. 1A) (*17*). Treatment of BCP-1 cells with hIL-6 or an irrelevant control protein, glutathione-S-transferase (GST), failed to reinitiate cell proliferation even though gp130 and gp80 receptors are constitutively expressed on these cells (fig. S1). The proliferative properties of vIL-6 on PEL cells in the absence of IFN-α were demonstrated by a 25-fold increased [^3H]thymidine uptake in vIL-6–treated cells compared with cells treated with GST alone. A similar but less pronounced effect [four- to fivefold increased uptake (*18*)] occurs when PEL cells are treated with vIL-6 in the presence of 20% fetal calf serum. Direct evidence for vIL-6 antagonism of IFN signaling was obtained from electrophoretic mobility–shift assays in which vIL-6 treatment blocked IFN-stimulated gene factor 3 (ISGF3) binding to IFN-stimulated response element (ISRE) probes, whereas hIL-6 had no effect (fig. S2). vIL-6 blockade of IFN signaling appears to be mediated through disruption of IFN receptor (IFNaR1 or IFNaR2) phosphorylation of Tyk2 kinase, leading to decoupling from the JAK-STAT signaling pathway (*18*). IFNs initiate G_1/S cell cycle arrest in part by inducing the cyclin-dependent kinase inhibitor $p21^{CIP1/WAF1}$ through an ISRE enhancer present in the $p21^{CIP1/WAF1}$ promoter (*19*). IFN-α induction of $p21^{CIP1/WAF1}$ in BCP-1 cells after 16 hours was blocked by vIL-6 treatment but not by hIL-6 treatment (Fig. 1B). This is achieved by interruption of IFN signaling, because no such effect is seen when cells are treated with doxorubicin to activate the $p21^{CIP1/WAF1}$ promoter through a p53-dependent pathway (*20*).

If vIL-6 itself is induced by IFN-α, then vIL-6 secretion could serve as an effector arm for a negative-feedback loop. IFN-α treatment of PEL cell lines results in a rapid up-regulation of vIL-6 transcription in both BCP-1 [KSHV-positive, Epstein-Barr virus (EBV)–negative] and BC-1 (KSHV-positive, EBV-positive) cell lines (fig. S3). vIL-6 is also induced by 12-*O*-tetradecanoylphorbol 13-acetate (TPA), a phorbol ester that initiates lytic transcription through de novo synthesis of the lytic transactivator protein Rta (ORF50) (*21*, *22*). Because IFN treatment can initiate lytic virus replication (*23*), up-regulation of vIL-6 might be secondary to lytic virus replication rather than a direct effect of IFN signaling. To distinguish between these two possibilities, we treated BCP-1 cells with TPA or IFN-α in the presence of the protein synthesis inhibitor cycloheximide (CHX). CHX completely blocks Rta-induced vIL-6 transcription, whereas IFN-α–induced vIL-6 transcription is only minimally inhibited (Fig. 2A).

Additional evidence that transcriptional regulation of vIL-6 is unique comes from cDNA hybridization of KSHV gene probes arrayed on nitrocellulose membranes. mRNAs harvested in triplicate from BCP-1 cells, with and without CHX pretreatment, were compared to mRNAs from cells treated for 12 hours with 500 U/ml of IFN-α in the presence or absence of CHX (Fig. 2B). Of the 89 KSHV and 7 control gene probes examined (table S1), only vIL-6 transcription was activated (average 30-fold up-regulation) by IFN-α in the presence of CHX. Two other viral genes, *ORF17* and *ORF62*, reached a threefold or greater induction 12 hours after IFN treatment, but both were blocked by CHX pretreatment. As expected, prolonged exposure to IFN-α (48 hours) resulted in activation of multiple viral genes because the virus enters lytic replication through secondary transcriptional cascades (*18*). These results show that additional pathways, beyond the dichotomous latency-lytic pathways traditionally described for herpesviruses, regulate vIL-6 gene transcription.

The vIL-6 promoter has two potential ISRE sequences; ISRE-1 is located −509 to −496 base pairs (bp) upstream of the vIL-6 translation start site [nucleotide position 17,875 (*24*)] and ISRE-2 is located at −420 to −401 bp. Sequential deletions of the vIL-6 promoter and site-directed mutagenesis of ISRE-1 or ISRE-2 demonstrated that both elements are required for maximal IFN-α–responsiveness of the promoter in BCP-1 cells (Fig. 3A).

To confirm that vIL-6 acts in an autocrine manner to block the growth-inhibitory effects of IFN, we reconstituted vIL-6 signaling in a heterologous cell line. Gene cassettes containing the vIL-6 coding region under the control of its native promoter, or promoters mutated at ISRE-1 and ISRE-2 sites, were stably introduced into IFN-sensitive, gp130-expressing Daudi C-11 cells that were not infected with KSHV (*25*). Basal levels of vIL-6 production between clones were comparable, suggesting similar gene dosages of the vIL-6 expression cassette (*18*). C-11 cells expressing vIL-6 from its wild-type promoter

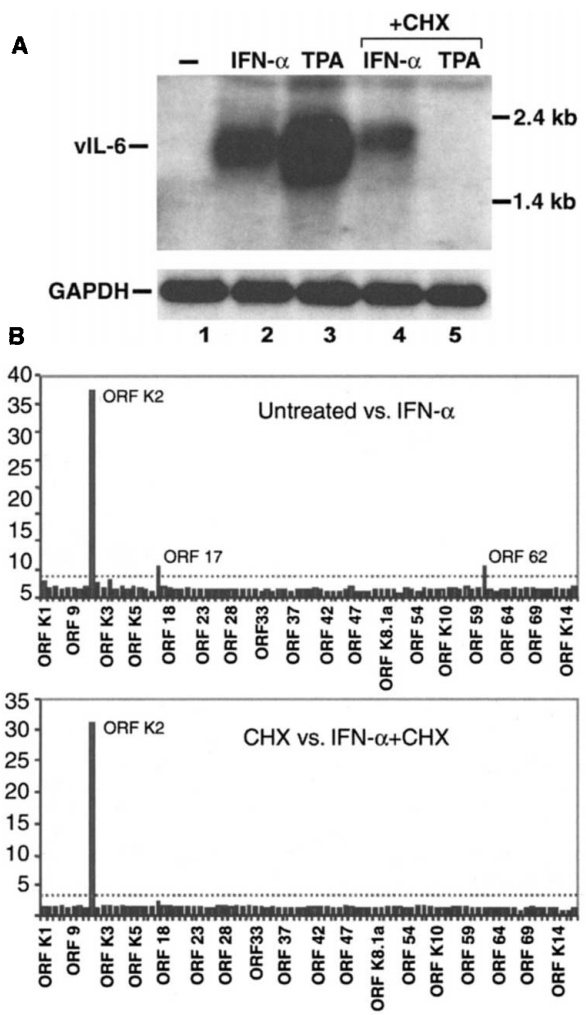

Fig. 2. The vIL-6 *ORFK2* gene is induced by IFN-α in an immediate-early fashion. (**A**) Northern blotting for vIL-6 expression using polyadenylated-selected mRNA from BCP-1 cells shows vIL-6 gene activation after 12 hours of treatment with either 500 U/ml IFN-α (lane 2) or 20 ng/ml TPA (lane 3). IFN-α induction (lane 4) is minimally affected by 30 min of pretreatment with 50 μg/ml CHX, whereas TPA induction (through synthesis of the KSHV ORF50 lytic transactivator) is abolished (lane 5). Reprobing with glyceraldehyde phosphate dehydrogenase (GAPDH, lower panel) served as a control for loading. (**B**) *ORFK2* encoding vIL-6 is the only immediate-early KSHV transcript induced by IFN-α. Polymerase chain reaction products for 89 KSHV genes were arrayed on nitrocellulose membranes and hybridized to cDNA from BCP-1 cells. (Top) Fold gene activation of untreated cells compared with cells treated for 12 hours with 500 U/ml of IFN-α. (Bottom) Fold gene activation at 12 hours with 500 U/ml of IFN-α when cells are pretreated with CHX (50 μg/ml for 30 min) to inhibit de novo protein synthesis. The dotted line shows the threefold induction level.

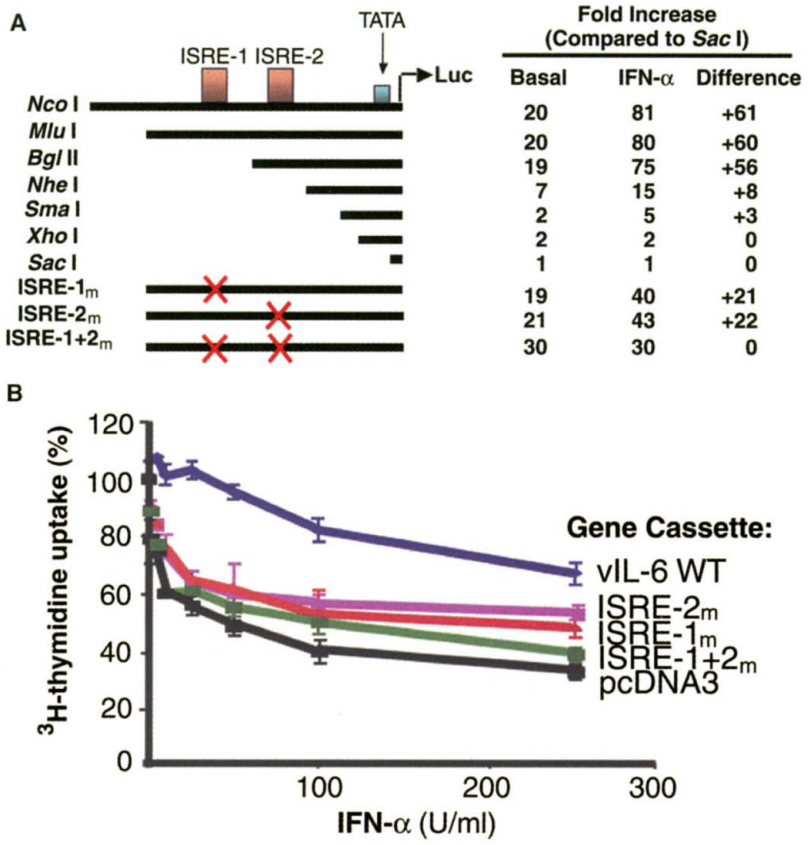

Fig. 3. Promoter activation by IFN-α allows KSHV cells to proliferate in the presence of IFN-α. (**A**) IFN-α activates the vIL-6 promoter in BCP-1 cells through two ISRE sequences in its promoter. The 761-bp Nco I vIL-6 promoter fragment, or sequential deletions of ISRE-1 (Bgl II fragment) and ISRE-2 (Nhe I fragment), were cloned into pGL3-basic luciferase reporter vector and treated with IFN-α (500 U/ml for 48 hours). Mutations in both ISRE-1 and ISRE-2 abolish IFN responsiveness of the full-length promoter. (**B**) Endogenous expression of vIL-6 through its promoter protects heterologous, KSHV– C-11 Daudi cells from IFN-α–induced proliferation arrest. IFN-α dose-response curves are shown for [^3H]thymidine uptake in C-11 Daudi cells stably selected for vIL-6 gene cassettes containing wild-type (WT) or mutant (m) promoters or empty vector (pcDNA3). C-11 Daudi cells containing the WT promoter vIL-6 gene cassette are resistant to IFN-α compared to cells with mutations in ISRE-1, ISRE-2, or both. Basal expression was comparable in all cell lines, indicating a similar gene dosage for all conditions (*18*).

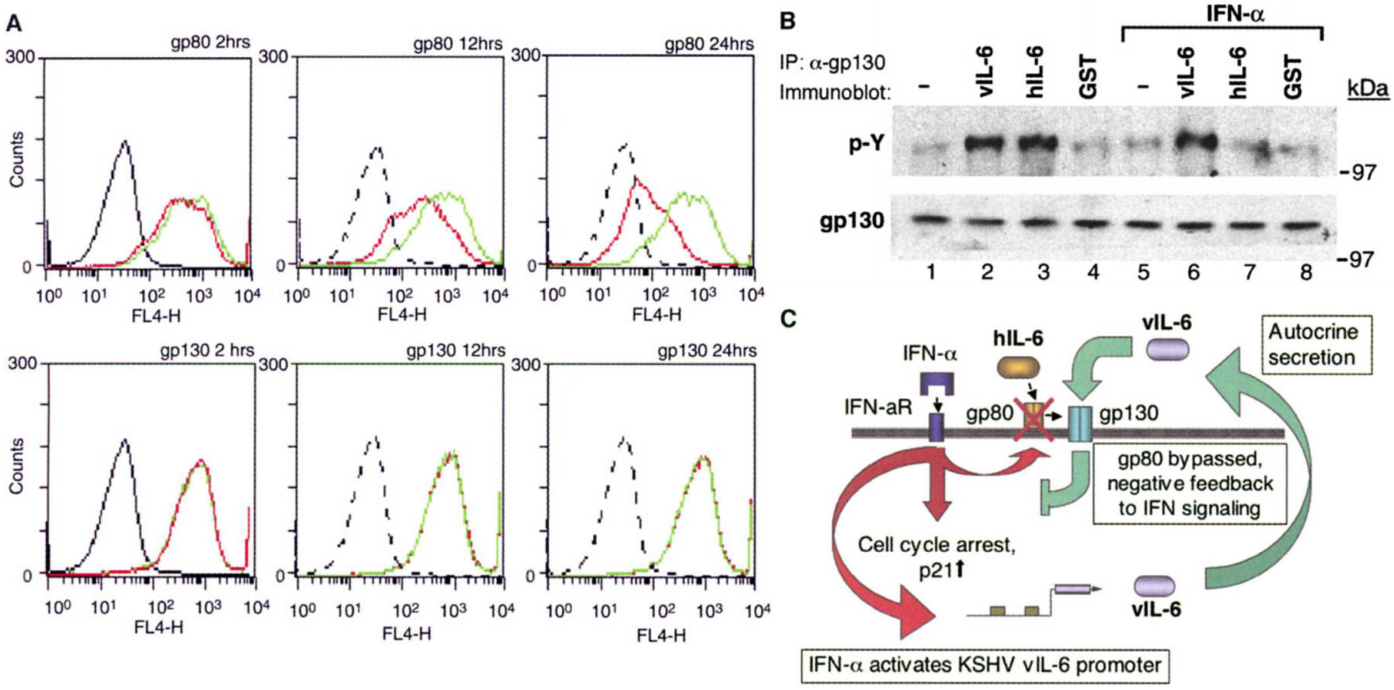

Fig. 4. Differences in vIL-6 and hIL-6 inhibition of IFN-α signaling are due to down-regulation of IL-6 gp80 receptor. (**A**) Flow cytometry for cell surface protein expression; increasing fluorescence intensity (FL4-H) plotted against cell counts. IFN-α (500 U/ml, green) down-regulates surface expression of the IL-6 receptor gp80 (upper panel) but not the signal transducer protein gp130 (lower panel), compared with untreated BCP-1 cells (red). Isotype secondary antibody control is shown in black. (**B**) hIL-6–induced gp130 phosphorylation is inhibited by IFN-α. BCP-1 cells were treated with 100 ng/ml each of vIL-6, hIL-6, or GST (lanes 2 to 4); 500 U/ml IFN-α alone (lane 5); or 500 U/ml IFN-α together with 100 ng/ml of vIL-6, hIL-6, or GST for 16 hours (lanes 6 to 8). Immunoprecipitates of gp130 from these cells were then immunoblotted for phosphotyrosine (p-Y); membranes were stripped and reblotted for gp130. IP, immunoprecipitate. (**C**) Model for KSHV vIL-6 inhibition of IFN-α signaling, resulting in cellular autocrine dependence on vIL-6.

were resistant to the growth-inhibitory effects of IFN-α and proliferated in the presence of 50 U/ml of IFN-α at a rate comparable to that of untreated controls (Fig. 3B). Cells possessing mutations in the vIL-6 promoter at either ISRE-1 or ISRE-2 had diminished IFN resistance and reduced proliferation at low concentrations of IFN-α.

Feedback inhibition of IFN signaling by vIL-6 provides a clear example of how virus subversion of host cell defenses can lead to cell proliferation. Why does cellular IL-6 not achieve the same effect? Both hIL-6 and vIL-6 can initiate IL-6 signaling in BCP-1 cells, as measured by electrophoretic mobility–shift assays in which the gamma-inteferon activation sequence (GAS) element from the interferon regulatory factor 1 (IRF-1) promoter is used as a probe, although vIL-6 signaling is more robust (*18*). The answer may lie in differences in receptor usage by the two cytokines. IFN-α treatment results in down-regulation of gp80 surface expression but has no effect on gp130 surface expression (Fig. 4A), an effect previously noted for other B cell lines, including the IL-6–dependent U266 multiple myeloma cell line (*26*). IFN-α also blocks hIL-6–induced but not vIL-6–induced gp130 tyrosine phosphorylation (Fig. 4B), demonstrating that the blockage occurs at the receptor level. gp80 mRNA expression is not markedly altered by IFN-α treatment, suggesting that gp80 blockade is largely due to posttranscriptional inhibition (fig. S4). This leads to a model (Fig. 4C) in which viral evolution has generated a modified cytokine that escapes regulatory control of IL-6 signaling by IFN-α. Infected cells that normally would either arrest or undergo apoptosis in response to IFN signaling continue to proliferate in the presence of vIL-6, resulting in a virus-human autocrine feedback circuit.

vIL-6 inhibits tumor-suppressor pathways activated during immune signaling, but it is important to emphasize that this is not solely responsible for PEL tumorigenesis, which results from multiple, combined viral and host cell genetic influences. Viruses have evolved a variety of ways to overcome host defenses against infection, including abrogating IFN signaling pathways (*27*, *28*). KSHV itself possesses another protein, vIRF-1, to inhibit IFN-mediated transcription. By sensing levels of IFN-α signaling, KSHV reacts to and modifies its cellular environment through vIL-6, exhibiting a fundamental property of biological systems called irritability that has been previously used to distinguish viruses from higher forms of life (*29*). In addition to immune evasion, it is possible that this mechanism plays a role in maintaining viral latency by preventing IFN induction of lytic replication. The autocrine loop established by vIL-6 illustrates mechanistically how interference with antiviral defenses can contribute to tumor cell proliferation and provides an attractive target for novel therapies directed against KSHV-related hematopoietic tumors.

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Supporting Online Material

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Figs. S1 to S4
Table S1
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53BP1, a Mediator of the DNA Damage Checkpoint

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53BP1 binds to the tumor suppressor protein p53 and has a potential role in DNA damage responses. We used small interfering RNA (siRNA) directed against 53BP1 in mammalian cells to demonstrate that 53BP1 is a key transducer of the DNA damage checkpoint signal. 53BP1 was required for p53 accumulation, G_2-M checkpoint arrest, and the intra-S-phase checkpoint in response to ionizing radiation. 53BP1 played a partially redundant role in phosphorylation of the downstream checkpoint effector proteins Brca1 and Chk2 but was required for the formation of Brca1 foci in a hierarchical branched pathway for the recruitment of repair and signaling proteins to sites of DNA damage.

53BP1 was identified through its ability to bind to the tumor suppressor protein p53 through 53BP1's COOH-terminal BRCT (Brca1 carboxyl-terminus) repeats (*1*, *2*), which are found in many DNA damage response proteins (*3*–*8*). 53BP1 responds to DNA double-strand breaks (*9*–*12*), quickly relocalizing to discrete nuclear foci upon exposure to ionizing radiation (IR). These foci colocalize with those of the Mre11-Nbs1-Rad50 complex and phosphorylated γ-H2AX, which are thought to facilitate the recruitment of repair factors to damaged DNA (*9*–*11*). In response to IR, 53BP1 is phosphorylated in an ataxia telangiectasia mutated (ATM)–dependent manner (*10*–*12*), but its role in the DNA damage response is unclear.

To determine 53BP1's role, we used small interfering RNAs (siRNAs) in the form of two independent, nonoverlapping, 21–base pair RNA duplexes that target 53BP1 to inhibit its expression (*13*, *14*). U2OS cells were transfected with these siRNA oligonucleotides (oligos) and, within 3 days posttransfection, a portion of cells had undergone cell death (fig. S1). A similar phenotype was also observed in two other cell lines, Hct116 and Saos2 (*15*).

To determine whether 53BP1 plays a role in DNA damage cell cycle checkpoints, we examined the response of 53BP1-inhibited cells to IR. IR induces the intra-S-phase

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