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Genes affecting ionizing radiation survival identified through combined exome sequencing and functional screening

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Abstract

The study of genetic syndromes characterized by sensitivity to DNA damaging agents has provided important insights into the mechanisms that maintain genome stability and identified novel targets for cancer therapies. Here, we used exome sequencing to study 51 unrelated individuals with previously reported hypersensitivity to ionizing radiation as well as a range of neurologic, immunologic, and developmental features, but who did not clearly fit any previously defined genetic syndrome. Based on the combination of variant identification, computational evidence of deleteriousness, and functional screening, we identified three groups of subjects. Two subjects carried the bi-allelic loss of function variants in causative genes for known DNA damage response syndromes. Eight subjects carried the single loss of function variants in causative genes for DNA damage response syndromes, six of whom also carried predicted deleterious variants in other genes with DNA damage-related functions. Three subjects carried deleterious mutations in genes without obvious roles in DNA damage responses. However, treatment of U2OS cells with small interfering RNA targeting these genes resulted in significantly increased radiation sensitivity. Our results suggest that gene–gene interaction may contribute to ionizing radiation sensitivity as well as highlighting possible roles for several genes not obviously involved in the response to DNA damage.

KEYWORDS
cellular survival, DNA damage disorders, genetic variants, radiation hypersensitivity, whole-exome sequencing

1 | INTRODUCTION

Since the pioneering work of Hermann Muller, it has been recognized that ionizing radiation is a potent mutagen (Muller, 1927). DNA double-strand breaks, the primary lesions induced by ionizing radiation, are particularly toxic because they constitute a failure of genome integrity rather than a simple loss or alteration of genetic information. Therefore, an understanding of cellular responses to ionizing radiation exposure can inform broadly about mechanisms critical for maintaining genome stability, which, when abrogated, can lead to malignancy.

In humans, much of our understanding of the biochemical pathways involved in DNA damage response (DDR) has come through genetic studies of rare recessive disorders characterized by

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sensitivity to one of a number of DNA damaging agents. Several such genetic disorders that include hypersensitivity to ionizing radiation as a clinical feature have been identified, largely because radiation hypersensitivity tends to co-occur with an elevated incidence of cancer, as well as with a constellation of other neurologic, developmental, or immune-related features (R. A. Gatti et al., 2007; Mizutani & Takagi, 2013). The study of these disorders has identified genes that affect ionizing radiation sensitivity and, in some cases, also act as moderate to high penetrance cancer risk genes in the general population. A prime example is the serine-threonine kinase *ATM* in which biallelic mutations lead to the recessive disorder Ataxia-telangiectasia (A-T) (Savitsky et al., 1995), while heterozygous mutations significantly increase the risk for breast and other cancers (Renwick et al., 2006; Swift et al., 1987). Somatic mutations in *ATM* and other radiation-responsive genes also contribute to cancer risk or progression (Schaffner et al., 2000; Stankovic et al., 2001; Stilgenbauer et al., 1997; Stoppa-Lyonnet et al., 1998; Vorechovsky et al., 1997). Thus, while the known genetic disorders of DDR are rare, their impact can be outsized given the potential for cancer predisposition in heterozygous carriers, the effects of somatic mutations on cancer progression, and the utility of the genes identified as targets for novel therapeutic treatments for cancer (Duan et al., 2014; Liu et al., 2018; Stoepker et al., 2015).

While the approach of studying rare subjects with unexplained radiation hypersensitivity has been an effective means of identifying genes with broad effects on DDRs and genome stability, success has come primarily from family studies or studies where multiple affected individuals with a consistent phenotype could be ascertained (R.A. Gatti et al., 1988; Martin et al., 2014; O'Driscoll et al., 2001; Savitsky et al., 1995; G. S. Stewart et al., 1999; Varon et al., 1998). In the current study, we focus on a historical collection of unrelated individuals whose cells displayed radiation sensitivity defined by laboratory testing at a single clinic. The clinical presentations of these subjects, while broadly falling into the neurologic, developmental, and immunologic categories that have been associated with recessive DDR disorders, are diverse in their severity and manifestation. To identify genes associated with radiation sensitivity we utilized cell lines established from these subjects, applying whole-exome sequencing (WES) for variant identification, computational analyses and manual curation for variant prioritization, and functional testing for validation.

2 | METHODS

2.1 | Editorial policies and ethical considerations

This study was approved by the Institutional Review Board (IRB-01) at the University of Florida (Study #IRB201400444). Informed consent, allowing for future research-based genetic studies, was obtained from all subjects at the time of initial clinical radiation sensitivity testing. Coded cell lines, derived from these subjects, were utilized for the current genomic studies described here.

2.2 | Subjects

The B-lymphoblastoid cell lines studied were previously established using blood samples from subjects originally referred for clinical testing for A-T or Nijmegen breakage syndrome (NBS) (X. Li et al., 2012; Nahas et al., 2012). Prior clinical testing, carried out over the previous 25 years, included clonogenic survival assays performed on the B cell lines to measure radiation sensitivity as well as DNA-based screening that excluded causal mutations in the *ATM* and *NBN* genes (Huo et al., 1994; Nahas et al., 2012). The results of clonogenic survival assay data from B-lymphoblastoid cell lines established from healthy controls and A-T patients, as well as subjects RS7, RS8, RS12, RS14, RS18, RS31, RS47, RS63, RS64, and RS68 have been previously published (Hu et al., 2017; Nahas et al., 2012). Clinical information was not systematically collected as part of the testing regimen, but those subjects for whom information was provided at referral displayed an array of overlapping clinical features consistent with A-T or NBS, including ataxia, microcephaly, other neurologic features, clinical immunodeficiency, growth retardation, and cancer at the time of referral (Table S1). All subjects studied were unrelated; biospecimens from additional family members were not available. As the original study is closed, no recontact of research subjects or access to their health information was possible.

2.3 | Whole-exome sequencing

The whole-exome paired-end sequencing was performed on an Illumina HiSeq using the Agilent Sureselect v1 exome capture kit (Agilent), which targeted approximately 37.6 Mb of the human genome with at least 30X coverage. FastQC (v0.11.2) was used to assess the quality of the raw sequencing data and all samples were within normal quality thresholds.

2.4 | Sequence alignment and variant detection

Sequencing reads were aligned to the human genome reference sequence (build GRCh37) using BWA-mem (H. Li & Durbin, 2009) (v0.7.12). Postalignment processing included duplicate marking using Samblaster (v0.1.21), SAM to BAM conversion, and BAM file sorting using Sambamba (v0.5.1). SAMtools (v1.2-192-gfcaafe0) was used for the analysis of BAM files. Base quality score recalibration was applied using Genome Analysis Toolkit (McKenna et al., 2010) (GATK; v3.3-0-g37228af). Finally, the GATK best practices workflow was used to identify single-nucleotide and insertion-deletion variation jointly among all samples.

2.5 | Postvariant calling quality control

Sample-level quality control was carried out to identify potential sample swaps and sequencing quality using Peddy (Pedersen &

Quinlan, 2017). Peddy was also used to infer and confirm sample ancestry, relatedness, and sex. Sequencing coverage for target regions was calculated with bedtools (Quinlan & Hall, 2010). GNU parallel (Tange, 2011) was used to run this analysis on all samples. The results were plotted using R software to visualize the sequencing coverage and assess power for variant discovery for each sample.

2.6 | Variant annotation

All identified genetic variants were annotated with predicted functional consequences and informative variant statistics, including Polyphen2 (Adzhubei et al., 2013), SIFT (Kumar et al., 2009), CADD (Kircher et al., 2014), CCR (Havrilla et al., 2018), and pLI scores (Lek et al., 2016)) using the Variant Effect Predictor (McLaren et al., 2016) (VEP) v83. Variants were also annotated with allele frequencies across multiple populations and the maximum population allele frequency (Popmax-AF) from the Genome Aggregation Database (Karczewski et al., 2019) (gnomAD) using vcfAnno (Pedersen et al., 2016). All the annotations were integrated using GEMINI (Paila et al., 2013).

2.7 | Variant prioritization

Variants were manually curated to identify subjects with loss of function variants in causative genes for established DDR disorders. A computational scan for variants occurring in any gene whose product acts in cellular DDR pathways (Table S2) was then carried out. The remaining variants were prioritized under the assumption of a recessive loss of function model using GEMINI (Paila et al., 2013) under two different schemes:

1. *Homozygous recessive model*. Rare or novel (with respect to gnomAD) homozygous recessive variants were identified using filtering criteria that required the variant to have a Popmax-AF of less than 0.0001, high or medium predicted impact on protein function, and homozygosity for the alternate genotype in at least one sample. For samples where no putative gene candidates were identified, the MAF thresholds were relaxed to 0.0005 or 0.001.
2. *Compound heterozygous recessive model*. Putative compound heterozygous variants were identified with GEMINI's "comp-hets" analysis tool. The population frequency and variant impact threshold employed were the same as for homozygous recessive variants, with the additional requirement of two or fewer heterozygous alleles across all samples. The Popmax-AF threshold was relaxed to as low as 0.001 for samples where no putative candidates were identified.

As an additional level of prioritization, the variants identified by each of these schemes were further scrutinized for their predicted deleteriousness using multiple informative metrics. Genes with predicted protein-truncating variants or variants predicted to be deleterious based on SIFT, PolyPhen2, pLI (>0.9), CCR (>0.95), or CADD scores (>15) were prioritized for further functional validation.

2.8 | Cell culture

B-lymphoblastoid cell lines established from radiosensitive subjects were maintained in Roswell Park Memorial Institute medium supplemented with 15% fetal bovine serum and 1% penicillin-streptomycin-glutamine. The SV40-transformed human fibroblast cell line, GM00637, was obtained from Coriell Institute for Medical Research (Camden, NJ) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine. The osteosarcoma cell line, U2OS, was obtained from ATCC and maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine. All cell lines were grown at 37°C, 5% CO_2, and 95% humidity.

2.9 | Antibodies and immunoblotting

Antibodies directed against human ATM (Novus Biologicals, Cat. no. 100-104), human FANCI (Bethyl Labs, Cat. no. A300-213A-T), and human NBN (Novus Biologicals, Cat. no. 100-143) were all rabbit polyclonals. A rabbit monoclonal antibody (Cell Signaling Technology, Inc., Cat. no. 4120) was used to detect MCPH1. All blots were probed with a monoclonal antibody directed against human γ-tubulin (Sigma, Cat. no. T5326) as a loading control. Immunoblotting was performed as previously described (Cerosaletti et al., 2006) with the exception that the sonication step was omitted in preparing cell lysates.

To estimate the increase in phosphorylation of SMC1 following irradiation, cells were mock-treated or irradiated at 3Gy and harvested 30 min later. Cell lysates were prepared, separated on sodium dodecyl sulfate-polyacrylamide gels, and blotted to nylon membranes as described (Cerosaletti et al., 2006). Immunoblots were sequentially probed with antibodies directed against human SMC1 phosphorylated on residue 957 (Cell Signaling Technology, Inc., Cat. no. 4802) and γ-tubulin. Blots were imaged and quantified with ImageQuant software (v8.1, Cytiva). The intensity of the bands for SMC1 was normalized to the tubulin loading control for each lane and a fold increase in normalized SMC1 signal was calculated by comparison of irradiated and mock-treated samples.

2.10 | Functional studies

Functional screening of computationally prioritized genes was carried out by a two-step process. In the first step, pools of three small interfering RNAs (siRNAs) per gene (Qiagen USA) were used to knockdown the expression of each of 77 candidate genes in triplicate in GM00637 cells. Knockdown cells were then irradiated at a dose of 4Gy and the overall viability of the bulk culture was assessed at 72 h postirradiation using

3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) staining. Results were compared to those obtained from GM00637 cells treated with the corresponding siRNAs but mock-irradiated. Mean survival and standard deviations were calculated using GraphPad Prizm v.9.1.

For the second step, clonogenic survival assays, U2OS cells were either mock-treated or treated in triplicate for 48 h with the same pools of three siRNAs used in the MTT assay. Cells, 250 to 2000 per well in a six-well plate, were then plated in McCoy's 5A medium and irradiated at doses of 0, 2, 4, and 6 Gy. After 3 weeks, colonies were fixed with 10% formaldehyde in phosphate-buffered saline (PBS), stained with 0.1% crystal violet in PBS and colonies of 80 cells or more were counted. Data were analyzed using CFAssay (http://www.bioconductor.org/packages/release/bioc/html/CFAssay.html) (Braselmann et al., 2015) and GraphPad Prizm (v.9.1). Briefly, colony counts were normalized to the numbers of cells plated. Means and standard deviations from triplicates were calculated and converted to surviving fractions by reference to the 0 Gy treatment point for each experiment. Linear-quadratic models were fit to the data. The analysis of variance *F*-test was used to assess the statistical significance of the differences between siRNA-transfected samples and control.

3 | RESULTS

3.1 | Whole-exome sequencing

WES was applied to DNA samples from 51 cell lines that had previously displayed impaired survival relative to normal controls after exposure to 2 Gy of ionizing radiation in clinical testing (Hu et al., 2017; Huo et al., 1994; Nahas et al., 2012). A summary of the testing results from clinical reports appears in Figure 1. Sequence coverage for WES target regions is provided in Figure S1; for the majority of samples, more than 65% of bases were sequenced to a depth of 30X. Three samples (RS31, RS56, and RS64) had less than 50% of captured bases covered at 30X depth and were excluded from subsequent analyses.

3.2 | Variants in causative genes for established DDR disorders

Established causative genes for rare recessive disorders characterized by radiation sensitivity were examined for deleterious variants to identify subjects with known DDR disorders. One subject, RS87, was likely to have Cockayne Syndrome B (Troelstra et al., 1992) based on biallelic frameshift variants in the *ERCC6* gene (Table 1).

A second subject, RS65, had a homozygous missense substitution in the gene *MCPH1* at a highly conserved position in the BRCT domain (Table 1). *MCPH1* is required for ionizing radiation-induced cell cycle arrest (Jackson et al., 2002; Lin et al., 2005) and biallelic loss of function mutations in *MCPH1* are the cause of a rare autosomal recessive disorder characterized by congenital microcephaly, mental retardation, misregulated chromosome condensation, and ionizing radiation sensitivity (Jackson et al., 2002; Trimborn et al., 2004). Although the variant detected was a missense substitution, no MCPH1 protein was detected by immunoblotting lysates from the RS65 cell line (Figure S2).

In addition to these instances of bi-allelic loss of function variants in DDR syndrome genes, we also observed a high frequency of heterozygous carriers of loss of function variants in causative genes for other rare, recessive DDR disorders, such as A-T, Fanconi anemia, and NBS (Table 1). Careful re-examination of the sequence data for these genes provided no evidence for a second, allelic deleterious variant in any of these individuals. We immunoblotted for the corresponding proteins in five of these subjects and all were detectable

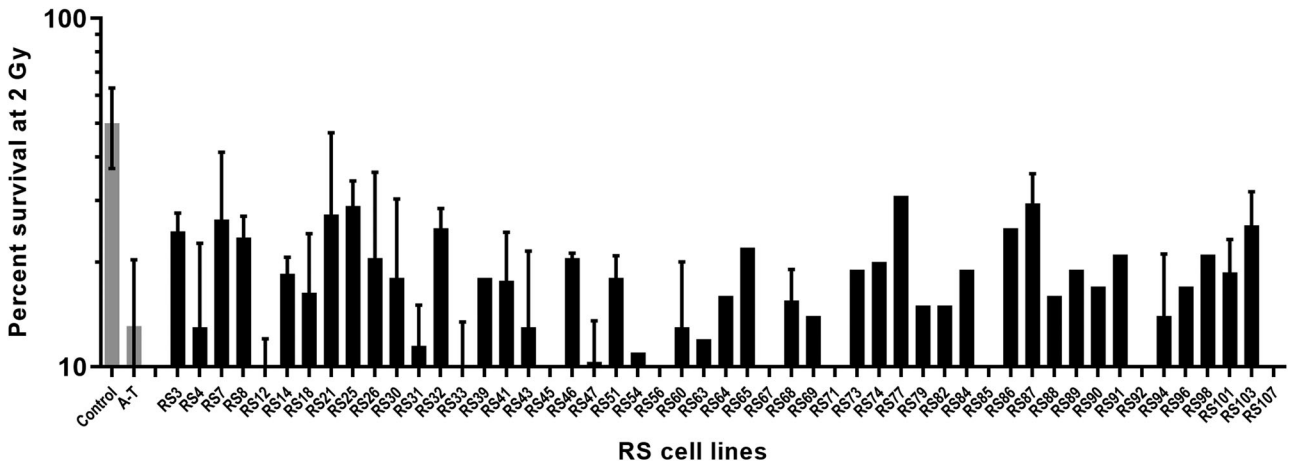

FIGURE 1 Clinical radiation sensitivity test results. Graph summarizes historical results of clinical clonogenic survival assays performed as described (Hu et al., 2017; Huo et al., 1994; Sun et al., 2002) but plotted on a $\log_{10}$ scale. Results from normal controls ($N = 29$) and A-T patients ($N = 104$) have been previously reported (Hu et al., 2017). Bars represent average percent survival relative to unirradiated cells and error bars indicate standard deviations. Numbers of tests for which results were available vary by subject, ranging from two to six

TABLE 1 Loss of function alleles in causative genes for recessive DNA damage response disorders

Subject	Gene	Mutations	Chromosomal position[a]	Disorder[b]
RS21	*ATM*	NM_000051.3:c.8545C>T, NP_000042.3:p.(Arg2849Ter)	11:108216595-108216596	Ataxia-telangiectasia
RS30	*BRCA2*	NM_000059.3:c.4593dup, NP_000050.2:p.(Val1532SerfsTer2)	13:32913078-32913079	Fanconi anemia
RS33	*FANCI*	NM_001113378.1:c.886G>T, NP_001106849.1:p.(Gly296Ter)	15:89816610-89816611	Fanconi anemia
RS46	*ATM*	NM_000051.3:c.1139_1142dup, NP_000042.3:p.(Ser381ArgfsTer27)	11:108119731-108119732	Ataxia-telangiectasia
RS65	*MCPH1*	NM_024596.5:c.236G>T, NP_078872.3:p.(Cys79Phe)	8:6289021-6289022	Primary microcephaly
RS67	*FANCA*	NM_000135.2:c.1615del, NP_000126.2:p.(Asp539Thrfs*66)	16:89849276-89849278	Fanconi anemia
RS73	*ATM*	NM_000051.3:c.1292del, NP_000042.3:p.(Glu431GlyfsTer6)	11:108121482-108121484	Ataxia-telangiectasia
RS73	*PNKP*	NM_007254.3:c.1295_1298+3del, NP_009185.2:p.?	19: 50365024-50365032	Ataxia-oculomotor apraxia 4
RS86	*NBN*	NM_002485.4:c.657_661del, NP_002476.2:p.(Lys219AsnfsTer16)	8:90983440-90983446	Nijmegen breakage syndrome
RS87	*ERCC6*	NM_000124.2:c.3607_3608insGGGCTGGCTGCTTAAGGTC-CACCTTA NP_000115.1:p.(Lys1203ArgfsTer33)	10:50678397-50678398	Cockayne syndrome B
		NM_000124.2:c.3591_3592dup, NP_000115.1:p.(Lys1198ArgfsTer4)	10:50678412-50678413	
RS90	*ATM*	NM_000051.3:c.7000_7003del, NP_000042.3:p.(Tyr2334GlnfsTer4)	11:108198390-108198395	Ataxia-telangiectasia

[a]Human genome build GRCh37.
[b]Disorders in which the indicated genes are known to be implicated.

at levels no less than half that of wild-type controls (Figure 2a). For three of the heterozygous *ATM* loss of function variant carriers, we also measured ATM kinase activity by immunoblotting for phosphorylation of SMC1, a known ATM substrate (Kim et al., 2002) following irradiation. The retention of radiation-inducible kinase activity in these samples together with detectable ATM protein argues against the presence of a second deleterious *ATM* variant that was not detected by exome sequencing (Figure 2b).

The detection of eight carriers of loss of function variants in causative genes for rare, recessive DDR disorders among a sample of only 48 subjects raises the possibility that haploinsufficiency at these loci, either alone, or in the context of other genetic variants, might contribute to their radiation-sensitive phenotype. To explore the possible contribution of multigenic inheritance to the phenotypes in these subjects, we searched for additional rare (MAF < 0.001) variants in genes encoding proteins involved in cellular DDR pathways (Table S2) in these subjects. Six of the eight carriers (Table 2) had at least one additional variant in a DDR gene that either would result in loss of function or was predicted to be probably damaging or deleterious by the programs Polyphen 2, or SIFT, respectively. The most notable example was the cell line RS73 that carried frameshift variants in both *ATM* and *PNKP* as well as a splice donor variant in Cyclin H (*CCNH*). Among the remaining 38 subjects, there were 31 with at least one variant in a DDR gene that was either loss of function or predicted as probably damaging or deleterious. Fourteen of these subjects had two or more such variants. In all cases, these occurred in distinct genes (Table S3).

3.3 | Computational prioritization of genes

After accounting for variants in causative genes for known DDR disorders, we assumed a recessive loss of function model in evaluating the remaining genetic variants identified. Such a model is consistent with the known Mendelian disorders characterized by radiation sensitivity. We further filtered variants on allele frequency on the expectation that, for the rare phenotypes represented in this cohort of subjects, the causative variants should be correspondingly rare. Table 3 indicates the median number of variants observed in all subjects along with the median and maximum number of rare variants prioritized. A complete list of genes meeting our selection criteria is provided in Table S4.

3.4 | Functional screening

Computationally prioritized genes were evaluated for their effects on radiation survival after siRNA knockdown in a fibroblast line, GM00637, with documented normal radiation sensitivity (O'Driscoll et al., 2001; Wright et al., 1998). The viability of GM00637 cells in

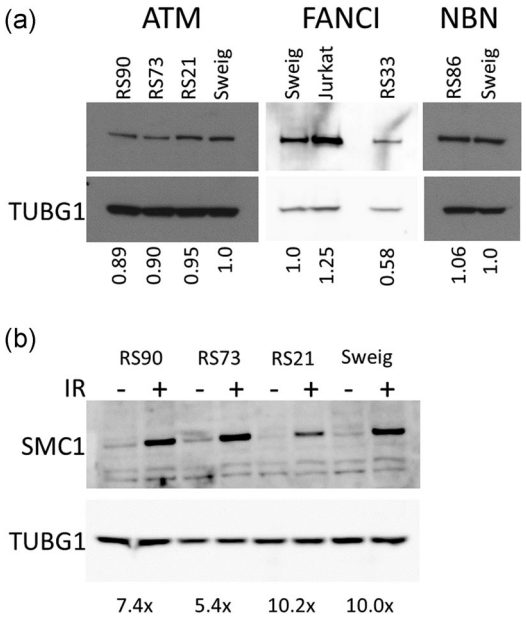

FIGURE 2 Protein expression and activity in carriers of loss of function mutations in genes for established DDR disorders. (a) Protein lysates prepared from B-lymphoblastoid cell lines established from carriers of loss of function mutations in *ATM*, *FANCI*, and *NBN* were electrophoresed on separate gels and immunoblotted with antibodies directed against the indicated proteins. A B-lymphoblastoid cell line with normal radiation sensitivity, Sweig, was included as a negative control on each gel. All blots were stripped and reprobed for γ-tubulin as a loading control. Signals were normalized to the tubulin controls, and are reported relative to the Sweig control on each immunoblot. (b) Cells from carriers of loss of function mutations in *ATM* were treated or mock-treated with 4 Gy of ionizing radiation (IR) as indicated by the + and − symbols. Protein lysates were prepared, electrophoresed, and immunoblotted with an antibody directed against the ATM phosphorylation site (S957) on SMC1. Comparably treated Sweig cells were included as a negative control. The calculated fold increase in SMC1 phosphorylation upon irradiation is indicated beneath each pair of samples

bulk culture after the individual siRNA knockdown of each of 77 individual prioritized genes (Table S5) and subsequent irradiation was assessed by staining with MTT (Figure 3). Results were compared to the same siRNA-treated cells that were sham irradiated. From the MTT assay results, we selected 13 genes for further study (Table 4) based on the criteria that their siRNA knockdown reduced viability after irradiation by more than one standard deviation below the mean for the collection of genes screened (Figure 3).

The 13 candidate genes selected from the MTT assay were subjected to a second, more stringent functional test for clonogenic survival of siRNA knockdowns in a second cell line, U2OS, exposed to a range of doses of ionizing radiation. Unlike the MTT assay, clonogenic assays measure the ability of individual cells to form colonies and are normalized to the simultaneously determined plating efficiency of the unirradiated cell line. This allows the direct measurement of the effects of radiation exposure on individual cell survival uncomplicated by the effects of cell cycle arrest, differential proliferation, or differential metabolism of the MTT dye. Of the 13 candidate genes in Table 4, only *CPSF1*, *DNAH3*, and *SPG11* passed this second round of functional screening, displaying dose-dependent impairment of radiation survival upon knockdown that differed significantly (*CPSF1*, $p = .001$; *DNAH3*, $p = .013$; *SPG11*, $p = .003$) from control irradiated U2OS cells without knockdown (Figure 4).

4 | DISCUSSION

Hypersensitivity to ionizing radiation has both biological and clinical relevance as it reflects underlying genetic instability and impacts the efficacy of a frequently used cancer therapy. Significant advances in our understanding of cellular DDR pathways have come from the identification of the underlying causative genes for recessive disorders characterized by radiation hypersensitivity. However, these approaches are limited by the rarity of the disorders and the inability to ascertain large numbers of subjects and/or families for classic linkage or association studies. Here, we demonstrate that even with single cases, the combination of exome sequencing, computational prioritization, and functional screening can identify candidate genes with roles in radiation sensitivity.

We studied a historical collection of subjects who shared sufficient clinical features with the known genetic disorders A-T or NBS to have been referred for radiation sensitivity testing, but who, upon genetic screening, lacked bi-allelic deleterious variants in the corresponding causative genes for these disorders. We previously identified a subject with RNF168 deficiency/RIDDLE syndrome from this collection (Devgan et al., 2011). We also described two subjects for which we had obtained DNA samples from multiple family members and were able to take advantage of the pedigree information to identify two genes with novel roles in radiation responses, *ATIC* and *MTPAP*, and elucidate their functional roles (Liu et al., 2018; Martin et al., 2014). These findings suggest that this collection of cell lines is a useful source for the identification of additional genes impacting upon radiation sensitivity. However, the remainder of the cell line collection was derived from unrelated individuals and there was limited clinical information available to cluster the subjects into meaningful groups that might correspond to novel syndromes. The absence of either biosamples or data from additional family members and the inability to recontact the probands required an alternative approach to identify potential causative genes.

Here, we describe a strategy combining computational prioritization of variants identified by WES with two orthogonal functional validation schemes. This approach allowed us to screen larger numbers of subjects and to identify likely causative variants in two genes with known roles in DDRs, *ERCC6*, and *MCPH1*. Although not normally associated with radiation sensitivity, *ERCC6* is part of the DNA excision repair pathway that removes bulky adducts from DNA, and the knockout of *ERCC6* in cell lines has been shown to confer radiation sensitivity (Batenburg et al., 2015). Deleterious variants in *MCPH1*, are associated with primary microcephaly. The majority of causative variants result in loss of function (Darvish et al., 2010; Trimborn et al., 2004). We detected a homozygous missense substitution in *MCPH1* but, upon immunoblotting, no corresponding MCPH1 protein was detected in the cell line from this subject.

TABLE 2 Variants in DNA damage response genes co-occurring with loss of function alleles in causative genes for recessive DNA damage response disorders

Subject	Gene	Variants	Impact[a]	Predicted effects[b]	
				Polyphen2	SIFT
RS21	***ATM***	**NM_000051.3:c.8545C>T, NP_000042.3:p.(Arg2849Ter)[c]**	**Stopgain**		
	RAD23A	NM_005053.4(RAD23A_v001):c.541G>C	Missense	Benign	Tolerated
		NM_005053.4(RAD23A_i001):p.(Val181Leu)			
	RNF8	NM_003958.3(RNF8_v001):c.113T>C	Splice region		
		NM_003958.3(RNF8_i001):p.(Val38Ala)			
	ERCC6	NM_000124.3(ERCC6_v001):c.3443A>T	Missense	Benign	Tolerated
		NM_000124.3(ERCC6_i001):p.(Asp1148Val)			
	RNF168	NM_152617.3(RNF168_v001):c.235A>G	Missense	Benign	Tolerated
		NM_152617.3(RNF168_i001):p.(Ile79Val)			
	ISG15	NM_005101.3(ISG15_v001):c.295C>T,	Missense	Benign	Tolerated
		NM_005101.3(ISG15_i001):p.(Arg99Trp)			
	UBA7	NM_003335.2(UBA7_v001):c.64C>G	Missense	Probably damaging	Deleterious
		NM_003335.2(UBA7_i001):p.(Leu22Val)			
RS30	***BRCA2***	**NM_000059.3:c.4593dup, NP_000050.2:p.(Val1532SerfsTer2)[c]**	**Frameshift**		
	BARD1	NM_000465.2(BARD1_v001):c.2179G>A	Missense	Benign	Tolerated
		NM_000465.2(BARD1_i001):p.(Asp727Asn)			
RS33	***FANCI***	**NM_001113378.1:c.886G>T, NP_001106849.1:p.(Gly296Ter)[c]**	**Stopgain**		
	HIST2H2BE	NM_003528.2(HIST2H2BE_v001):c.327G>C	Missense	Probably damaging	Deleterious
		NM_003528.2(HIST2H2BE_i001):p.(Lys109Asn)			
	HIST1H2BO	NM_003527.4(H2BC17_v001):c.347C>G	Missense	Possibly damaging	Deleterious
		NM_003527.4(H2BC17_i001):p.(Thr116Arg)			
RS46	***ATM***	**NM_000051.3:c.1139_1142dup, NP_000042.3:p.(Ser381ArgfsTer27)[c]**	**Frameshift**		
	ATR	NM_001184.3(ATR_v001):c.5266G>A	Missense	Probably damaging	Deleterious
		NM_001184.3(ATR_i001):p.(Val1756Met)			
	KDM4A	NM_014663.2(KDM4A_v001):c.3102G>C	Missense	Benign	Tolerated
		NM_014663.2(KDM4A_i001):p.(Glu1034Asp)			
	PER1	NM_002616.2(PER1_v001):c.2243C>T	Missense	Benign	Deleterious
		NM_002616.2(PER1_i001):p.(Pro748Leu)			
	POLR2E	NM_002695.3(POLR2E_v001):c.340G>A	Missense	Probably damaging	Deleterious
		NM_002695.3(POLR2E_i001):p.(Ala114Thr)			
	XRCC6	NM_001469.3(XRCC6_v001):c.89A>G	Missense	Benign	Deleterious
		NM_001469.3(XRCC6_i001):p.(Tyr30Cys)			
RS67	***FANCA***	**NM_000135.2:c.1615del, NP_000126.2:p.(Asp539Thrfs*66)[c]**	**Frameshift**		
RS73	***ATM***	**NM_000051.3:c.1292del, NP_000042.3:p.(Glu431GlyfsTer6)[c]**	**Frameshift**		
RS73	***PNKP***	**NM_007254.3:c.1295_1298+3del, NP_009185.2:p.?[c]**	**Frameshift**		
	CHAF1	NM_005483.2(CHAF1A_v001):c.707A>G	Missense	Unknown	Tolerated
		NM_005483.2(CHAF1A_i001):p.(Lys236Arg)			

(Continues)

TABLE 2 (Continued)

Subject	Gene	Variants	Impact[a]	Predicted effects[b]	
				Polyphen2	SIFT
	RIF1	NM_001177663.1(RIF1_v001):c.5705A>G	Missense	Benign	Tolerated
		NM_001177663.1(RIF1_i001):p.(Asn1902Ser)			
	SIRT6	NM_016539.1(SIRT6_v001):c.502G>A	Missense	Benign	Tolerated
		NM_016539.1(SIRT6_i001):p.(Val168Met)			
	CCNH	NC_000005.9:g.86697517A>G	Splice donor		
RS86	***NBN***	**NM_002485.4:c.657_661del, NP_002476.2:p.(Lys219AsnfsTer16)[c]**	**Frameshift**		
	ACTB	NM_001101.3(ACTB_v001):c.180C>G	Missense	Probably damaging	Deleterious
		NM_001101.3(ACTB_i001):p.(Ser60Arg)			
	ERCC3[a]	NM_000122.1(ERCC3_v001):c.1130A>T	Missense	Probably damaging	Deleterious
		NM_000122.1(ERCC3_i001):p.(Gln377Leu)			
	ERCC3[a]	NM_000122.1(ERCC3_v001):c.1078C>T	Missense	Probably damaging	
		NM_000122.1(ERCC3_i001):p.(Arg360Cys)			
	NSD2	NM_001042424.2(NSD2_v001):c.628A>G	Missense	Benign	Tolerated
		NM_001042424.2(NSD2_i001):p.(Thr210Ala)			
RS90	***ATM***	**NM_000051.3:c.7000_7003del, NP_000042.3:p.(Tyr2334GlnfsTer4)[c]**	**Frameshift**		
	ATM	NM_000051.3(ATM_v001):c.7499T>C	Missense	Possibly damaging	Deleterious
		NM_000051.3(ATM_i001):p.(Val2500Ala)			
	ACTR5	NM_024855.3(ACTR5_v001):c.1717A>T	Missense	Benign	Tolerated
		NM_024855.3(ACTR5_i001):p.(Ile573Phe)			
	ASCC1	NM_001198798.2(ASCC1_v001):c.779G>A	Missense	Benign	Tolerated
		NM_001198798.2(ASCC1_i001):p.(Arg260His)			
	CCNA1	NM_001111046.1(CCNA1_v001):c.1060A>G	Missense	Benign	Tolerated
		NM_001111046.1(CCNA1_i001):p.(Thr354Ala)			
	HIST1H2BC	NM_003526.2(HIST1H2BC_v001):c.349A>T	Stopgain		
		NM_003526.2(HIST1H2BC_i001):p.(Lys117*)			
	XRCC4	NM_003401.3(XRCC4_v001):c.719A>G	Missense	Benign	Tolerated
		NM_003401.3(XRCC4_i001):p.(Gln240Arg)			
	RIF1	NM_001177663.1(RIF1_v001):c.2243C>T	Missense	Benign	Tolerated
		NM_001177663.1(RIF1_i001):p.(Ser748Phe)			
	PMS1	NM_000534.4(PMS1_v001):c.1328A>G	Missense	Benign	Tolerated
		NM_000534.4(PMS1_i001):p.(Asn443Ser)			

[a]These variants occur on the same allele.

[b]Where blank, no prediction is provided by the indicated software program.

[c]Indicates the corresponding heterozygous loss of function variant from Table 1.

This might be due to the nonconservative nature of the amino acid substitution occurring at a critical position in a functional domain, or an effect of the variant on splicing due to its proximity (+3 position) to a splice acceptor site.

We further identified three genes containing potentially damaging variants that, when knocked down by siRNA, rendered cells significantly more susceptible to the lethal effects of ionizing radiation. While our functional assays implicate these genes in

TABLE 3 Median number of protein-coding variants across 48 RS cell lines

Variant types	Observed variants (het; hom alt)[a] Median	Prioritized variants (het; hom alt) Median	Max
Missense	5043;3093.5	2;0.5	7;14
Synonymous	5484;3427.5	0;0	0;0
Frameshift	118;41	0;0	2;1
Splice acceptor	26.5;12	0;0	1;1
Splice donor	30.5;15	0;0	1;0
Start lost	7;4	0;0	0;0
Stop gained	55;12	0;0	2;1
Stop lost	10;8	0;0	0;0
Splice region variants	1496;724.5	0;0	2;2
In-frame deletion	68.5;26	0;0	1;0

[a](Heterozygous; homozygous for alternate allele).

radiosensitization, they do not address the other phenotypes present in these subjects. It remains possible that the neurologic, and other phenotypes in these subjects result from other genetic or environmental causes. The three genes have diverse roles. *SPG11* encodes a protein involved in the control of cell migration or proliferation, particularly in the nervous system, consistent with the presence of neurologic features in the subjects (Howard et al., 2016; Perez-Branguli et al., 2019; Pozner et al., 2020; Tan et al., 2019; Zhou et al., 2012). *CPSF1* encodes a component of the cleavage and polyadenylation complex that both affects alternative splicing and controls the specificity of polyadenylation (Bolli et al., 2011) (Evsyukova et al., 2013). *DNAH3* encodes a dynein heavy chain that forms part of a large multi-subunit ATPase that provides energy for cellular microtubule function (Milisav, 1998).

While none of these genes have been previously implicated in cellular sensitivity to ionizing radiation or, more broadly, in the human DDR, there is suggestive evidence for their potential roles. One of the earliest cellular responses to ionizing radiation exposure is the activation of the serine-threonine kinase ATM that, together with the related kinase, ATR, phosphorylate more than 800 different proteins that control cell proliferation, metabolism, and DNA repair (Bakkenist & Kastan, 2003; Kastan & Lim, 2000; Matsuoka et al., 2007). SPG11 is phosphorylated by ATM or ATR in response to radiation (Matsuoka et al., 2007). CPSF1 is not a reported substrate of ATM or ATR, but other related components of the cleavage and polyadenylation complex, such as CPSF6, are, linking the function of this complex, if not CPSF1 specifically, with the cellular response to DNA double-strand breaks. The protein products of *CPSF1*, *DNAH3*, and two other genes we had previously identified from family studies of radiation-sensitive subjects, *ATIC* and *MTPAP*, are all reported to interact with an E3 ubiquitin ligase, RNF123, which plays a role in DDRs through the turnover of the ATR kinase (Muralikrishna et al., 2012). In an RNAi screen for genes involved in ionizing radiation sensitivity or resistance, Hurov et al. (2010) identified 850 genes whose knockdown resulted in significant radiosensitization. Interestingly, these genes include other members of the dynein heavy chain (*DNAH17*) and cleavage and polyadenylation specificity factor families (*CPSF6*) as well as two additional genes in which we identified deleterious variants in our study, *DLL1* and *SLC38A10* (Hurov et al., 2010).

Most known genetic disorders characterized by radiation hypersensitivity are single-gene disorders (Martin et al., 2014; O'Driscoll et al., 2001; Savitsky et al., 1995; G. S. Stewart et al., 1999, 2009; Varon et al., 1998). We considered the possibility that, in some of the individuals studied here, two or more genes could act jointly to create a radiation-sensitive phenotype. We identified eight subjects who were heterozygous carriers of rare loss of function alleles in genes known to be responsible for human DDR disorders. Despite careful scrutiny of variant calls and testing for protein production and activity, there was no evidence for a second deleterious allele in the same gene in any of these subjects. In one case, FANCI in subject RS33, we did observe a reduction in protein level consistent with one allele either failing to be expressed or its product being degraded via nonsense-mediated decay. Given that the incidence rates for the corresponding disorders are all less than 1/100,000 live births, this detection of eight heterozygous carriers in 48 subjects is unlikely to be a chance event. There are reports of modest radiosensitivity among *ATM* heterozygous carriers measured by various in vitro assays, which might account for their increased representation in our study population (Neubauer et al., 2002; Pollard & Gatti, 2009; West et al., 1995). Alternatively, haploinsufficiency at DDR loci may act jointly with variants at other loci to modulate radiation sensitivity. Indeed, one subject, RS73, is a carrier of a single loss of function alleles at two DDR loci, *ATM* and *PNKP*, and also had a missense substitution at a conserved splice donor site in *CCNH*. A second subject, RS46, had loss of function alleles at both *ATM* and *CPSF1* as well as predicted deleterious missense variants in *ATR and POLR2E*. Four of the remaining six subjects carried additional variants predicted to be possibly damaging or deleterious in other genes with known roles in DDR pathways. These observations suggest the possibility that a subset of radiation-sensitive individuals may have genetically complex etiologies requiring more complicated genetic and functional modeling approaches for their elucidation.

There are several lines of evidence that suggest that DDRs can be impacted by the combined actions of more than one gene. Gatz et al. have described a subject with mutations in two genes whose products act in different biochemical pathways, resulting in defects in DNA repair and damage signaling (Gatz et al., 2016). Synthetic lethality approaches to cancer treatment in which tumor mutations in one DDR pathway are exploited by treatment with a drug inhibiting a target in a second pathway have been used to increase sensitivity to DNA damaging agents (Lord & Ashworth, 2017). Finally, in mouse models, the combination of loss of function variants in different DDR genes has been shown to impact survival (Lee et al., 2000).

In the current study, by applying exome sequencing and computational variant, prioritizing combined with targeted functional

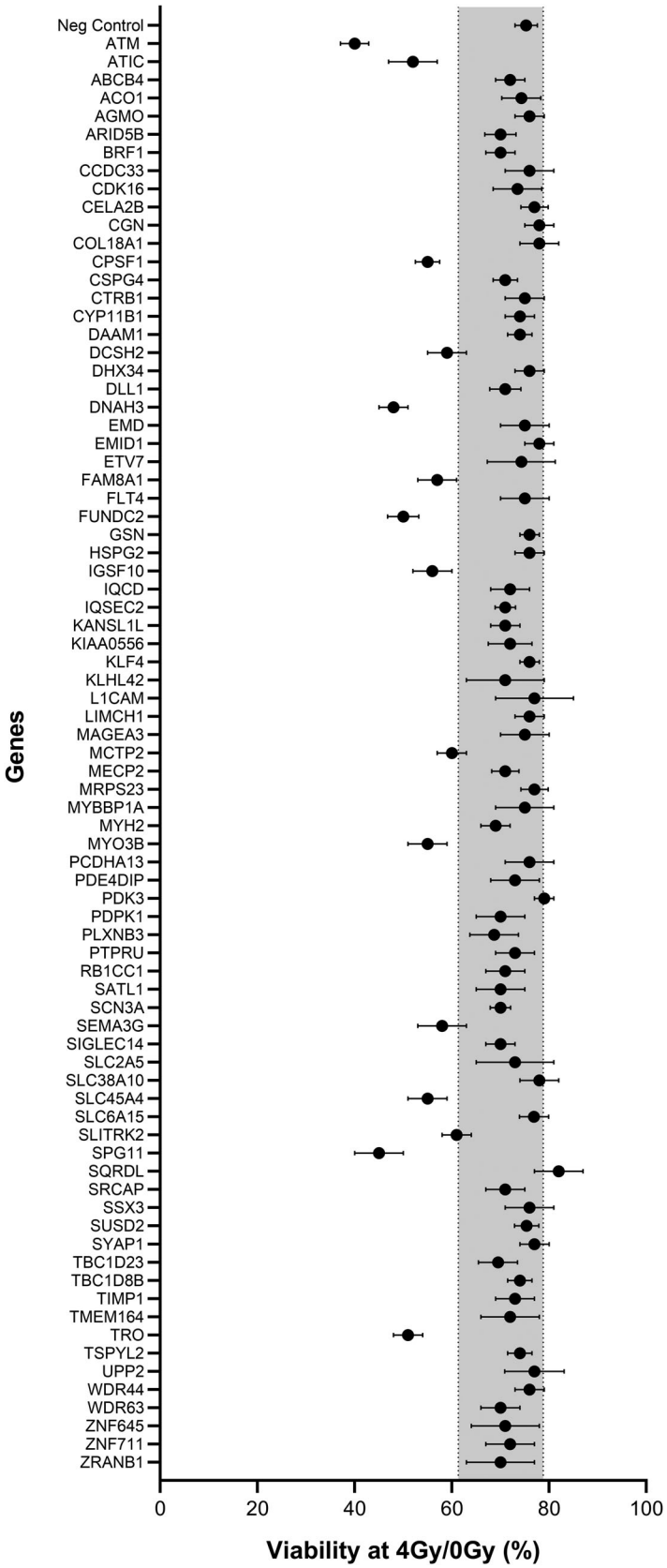

FIGURE 3 First round functional screen for radiation survival. *ATM* and *ATIC* serve as positive controls and nonsense RNAi as a negative control. Survival percentage indicates the difference in survival in cells treated with 4 Gy of ionizing radiation compared to sham treatment. Mean survival ± one standard deviation based on biological triplicates are indicated for each gene tested. Shading indicates the mean survival ± one standard deviation for all genes tested. Candidate genes whose knockdowns resulted in a reduction in survival of greater than one standard deviation below the mean for all genes tested were prioritized for further study and are listed in Table 4

TABLE 4 Genes selected for reduced radiation survival in the MTT assay

Gene	Gene full name	Mutations	Chromosomal position[a]
CPSF1[b]	Cleavage and polyadenylation-specific factor 1	c.2310_2325dup, p.(Pro776*)	8:145622761-145622762
		c.2636T>C, p.(His879Arg)	8:145622100-145622101
DCHS2	Dachsous cadherin-related 2	c.1323C>G, p.(His441Gln) c.7969G>A, p.(Glu2657Lys)	4:155254539-155254540 4:155156469-155156470
DNAH3[b]	Dynein, axonemal, heavy chain 3	c.2971T>G, p.(Leu991Val) c.5858G>A, p.(Gly1953Glu)	16:21086880-21086881 16:21031109-21031110
FAM8A1	Family with sequence similarity 8, member A1	c.509G>A, p.(Gly170Asp)	6:17601148-17601149
FUNDC2[c]	FUN 14 domain containing 2	c.158C>T, p.(Ser53Leu)	X:154261701-154261702
IGSF10	Immunoglobulin superfamily, member 10	c.2210_2211 del, p.(Phe737*) c.2321A>C, p.(Asn774Thr)	3:151165556-151165559 3:151165447-151165448
MCTP2	Multiple C2 domains, transmembrane 2	c.56T>A, p.(Leu19*)	15:94841549-94841550
MYO3B	Myosin IIIB	c.2521-2A>G, p.(?) c.2938A>T, p.(Thr980Ser)	2:171264222-171264223 2:171323144-171323145
SEMA3G	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3G	c.1078C>T, p.(Arg360*) c.551-8delC, p.(?)	3:52474457-52474458 3:52475712-52475714
SLC45A4	Solute carrier family 45 member 4	c.543G>T, p.(Gln181His) c.1162C>T, p.(Arg388Cys)	8:142229042-142229043 8:142228423-142228424
SLITRK2[c]	SLIT and NTRK-like family, member 2	c.2308G>C, p.(Gly770Arg)	X:144906250-144906251
SPG11[b]	Spastic paraplegia 11 (autosomal recessive)	c.6157G>A, p.(Val2053Met) c.6271C>T, p.(Gln2091*)	15:44865792-44865793 15:44864952-44864953
TRO[c]	Trophinin	c.2624C>T, p.(Thr875Met)	X:54955780-54955781

Abbreviation: MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide.

[a]Human genome build GRCh37.

[b]Genes that passed the second functional screen.

[c]X-linked gene in a male subject.

assays to a collection of unrelated subjects with prior evidence of cellular radiation hypersensitivity, we were able to identify two likely causative genes with established roles in DDRs, and three genes with previously unrecognized roles in human cellular responses to ionizing radiation. Support for these latter genes comes from both the detection of deleterious variants in these genes in radiosensitive individuals and from their ability to radiosensitize normal cells upon siRNA knockdown. Published data indicates that the products of several of these genes are either posttranslationally modified in response to radiation exposure or physically interact with other proteins involved in DDRs. Our findings also suggest the potential for more complex modes of inheritance that may involve haploinsufficiency and/or gene–gene interaction. There are, however, some limitations to the study design that impact the potential interpretation of data. To screen the large number of candidate genes identified from sequencing and computational analyses, we employed an assay based on MTT staining. This assay, which depends on cellular metabolism and proliferation, has the potential to generate false negatives. In this regard, we note that two genes, *DLL1* and *SLC38A10*, which we did not follow up based on the results of this assay, have been previously reported to radiosensitize upon knockdown in a genome-wide RNAi screen (Hurov et al., 2010) and three others, *BRF1*, *SRCAP*, and *WDR44* encode ATM/ATR phosphorylation substrates. Both of the functional assays we employed are dependent on the degree and specificity of siRNA knockdown achieved. Because of the large numbers of genes involved, and the need to individually validate antibodies to test for reductions in protein expression, we did not evaluate the success of knockdowns in these assays by immunoblotting. However, we did, in all cases, employ pools of three distinct siRNAs targeting each gene. Finally, we note that the starting point for our study is exome sequencing of cell lines derived from patients. The transformed nature of the cell lines, might, in some cases, impact their previously reported radiosensitivity. Exome data do not allow the detection of variants in introns or intergenic regions and have limited ability to support the detection of structural variants. Thus, there are classes of potential causative variants that would have been difficult to detect in the current study. We have deposited all of the exome sequencing data in a public database where other investigators can explore the role of mutations in the genes identified here or in other candidates as well as apply new analytic tools to identify and prioritize variants.

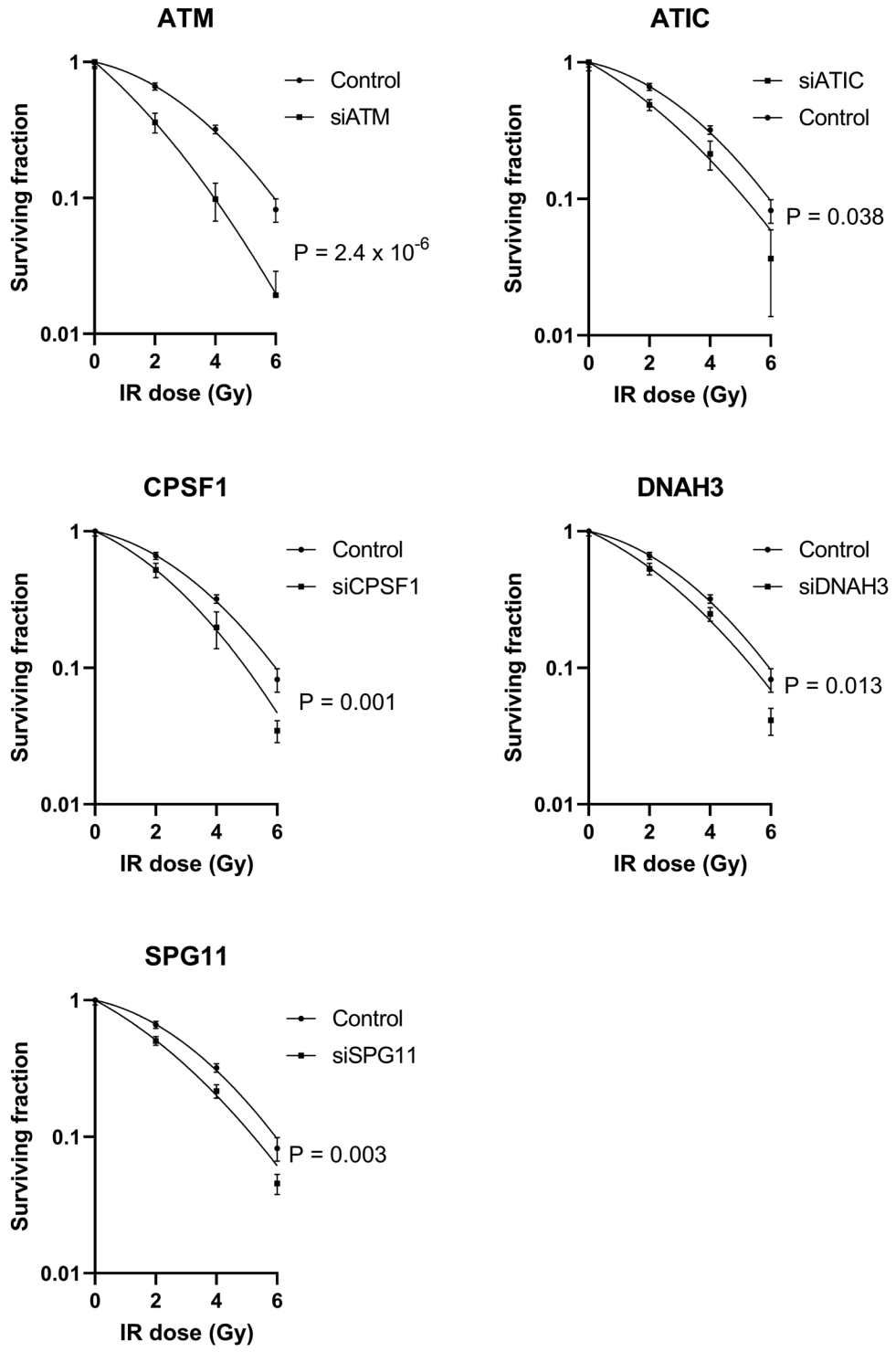

FIGURE 4 Clonogenic survival results for knockdown of candidate genes. Clonogenic survival curves for small interfering RNA (siRNA) knockdowns in U2OS cells of *ATM* and *ATIC* as positive controls and the candidate radiation response genes *CPSF1*, *DNAH3*, and *SPG11* (filled squares) are plotted relative to a survival curve for U2OS cells not exposed to siRNA (filled circles). Measurements were performed in triplicate and the mean surviving fraction ± standard deviation at each dose is plotted. Results for individual genes are plotted separately, but are part of a single large experiment with a single normal control not treated with siRNA

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CONFLICT OF INTERESTS

A. Q.Aaron Quinlan is a cofounder of Base2 Genomics, LLC, which licenses the GEMINI software used in this study. Patrick Concannon is a director of the Genetics Institute at the University of Florida and the institute has received event support from Illumina.

DATA AVAILABILITY STATEMENT

All raw and processed exome sequencing data generated in this study are available from the NCBI database of Genotypes and Phenotypes (dbGaP; http://www.ncbi.nlm.nih.gov/gap/) using the accession number phs001911.v1.p1.

WEB RESOURCES

Exome Aggregation Consortium (ExAC): http://exac.broadinstitute.org.

Genome Aggregation Database (gnomAD): https://gnomad.broadinstitute.org.

GeneCards DNA Double-Strand Break Repair SuperPath: https://pathcards.genecards.org/card/dna_double-strand_break_repair.

Human DNA Repair Genes: https://www.mdanderson.org/documents/Labs/Wood-Laboratory/human-dna-repair-genes.html.

CFAssay (http://www.bioconductor.org/packages/release/bioc/html/CFAssay.html).

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