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A Platform for Interrogating Cancer-Associated p53 Alleles

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Abstract

p53 is the most frequently mutated gene in human cancer. Compelling evidence argues that full transformation involves loss of growth suppression encoded by wild-type p53 together with poorly understood oncogenic activity encoded by missense mutations. Furthermore, distinguishing disease alleles from natural polymorphisms is an important clinical challenge. To interrogate the genetic activity of human p53 variants, we leveraged the *Drosophila* model as an *in vivo* platform. We engineered strains that replace the fly p53 gene with human alleles, producing a collection of stocks that are, in effect, 'humanized' for p53 variants. Like the fly counterpart, human p53 transcriptionally activated a biosensor and induced apoptosis after DNA damage. However, all humanized strains representing common alleles found in cancer patients failed to complement in these assays. Surprisingly, stimulus-dependent activation of hp53 occurred without stabilization, demonstrating that these two processes can be uncoupled. Like its fly counterpart, hp53 formed prominent nuclear foci in germline cells but cancer-associated p53 variants did not. Moreover, these same mutant alleles disrupted hp53 foci and inhibited biosensor activity, suggesting that these properties are functionally linked. Together these findings establish a functional platform for interrogating human p53 alleles and suggest that simple phenotypes could be used to stratify disease variants.

Keywords

mutant p53; gain-of-function p53; apoptosis; nuclear bodies

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Conflict of Interest

The authors declare no conflict of interest.

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Introduction

Over 50% of human cancers carry a mutation in the TP53 tumor suppressor gene(1). The p53 protein is a transcriptional hub, converting a diversity of stress-signals into adaptive programs such as cell-cycle arrest, DNA repair, or apoptosis(2). However, p53 clearly retains tumor suppressive activity in the combined absence of key apoptotic and cell cycle effectors (puma, noxa and p21) highlighting important gaps in our understanding of this network(3). Furthermore, unlike other tumor-suppressors which typically acquire inactivating frameshift and nonsense mutations, 75% of p53 mutations in human cancer are missense alleles with single-residue substitutions in the full-length protein(4). More than a third of all missense mutations occur in six residues in the DNA binding domain collectively called "hot-spot" residues. Although these mutations exhibit loss-of-function (LOF) effects in conventional assays, their high prevalence suggest a selective advantage over other LOF missense mutations. Furthermore, the substituted residues in hot-spot mutants undergo strong selection in cancers. Four of the hot-spot residues are predominantly substituted by a specific amino acid (R175H, G245S, R249S and R282W), whereas the other two residues are equally substituted by one of two amino acids (R248Q/W and R273C/H). Moreover, because full transformation of cancers harboring hot-spot mutations requires loss of the remaining wild-type allele (e.g. up to 60% in colon cancers(5)), their selective advantage is not fully explained by their dominant-negative activity on the wild-type allele(6, 7). Several elegant *in vivo* studies in mice have shown that these hot-spot mutations result in gain-of-function (GOF) activities - novel functions not characteristic of the wild-type allele. Specifically, mice harboring hot-spot mutations seen in cancer patients succumb to more invasive and metastatic cancers(6, 7) and even exhibit reduced survival when compared to $p53^{-/-}$ mice(8).

Several studies have advanced GOF activities for hp53 cancer alleles(9–11), but how these missense proteins exert their oncogenic activity is still largely unknown. In this manuscript, we establish *Drosophila* as a functional platform to interrogate human p53 variants *in vivo* and stratify mutations seen in the clinic. Since orthologs of *Mdm2, p63* and *p73* are absent from the fly genome, our platform presents unique opportunities to isolate intrinsic properties associated with hp53 cancer alleles.

Results and Discussion

Building humanized p53 strains

To generate a collection of humanized p53 fly strains, we used *in vitro* recombineering methods. Specifically, we targeted a BAC carrying a 20kb genomic fragment of the fly p53 locus and replaced the *Dp53* gene with a hp53 cDNA (Figure 1a). We then integrated this humanized genomic fragment into a molecularly defined region of the *Drosophila* genome, using site-directed recombination (Figure 1a). This strategy allowed preservation of all native fly p53 regulatory elements while permitting versatile genetic manipulation of the locus and efficient generation of transgenics. Using this approach, we successfully generated 8 transgenic lines (Figure 1b): one fly p53 rescue line ($Dp53^{+}$), 2 wild-type hp53 lines ($hp53^{1}$ and $hp53^{2}$) and 5 mutant hp53 lines which represent five of the most common hot-spot mutations in human cancer(12) (R175H, G245S, R248Q, R273C and R273H). The

mutant hp53 lines represent two categories of hp53 missense mutations(13): contact site mutations that affect residues which directly contact DNA without affecting the overall structure of the protein (R248Q and R273C/H); and conformational mutations which affect the structural conformation of the local DNA binding domain (R248Q, G245S) or the entirety of the protein (R175H). The R248Q mutant falls into both categories because it affects a contact site residue and perturbs the structure of the DNA binding domain(14). All of these transgenic lines were then crossed to a *dp53*$^{-/-}$ background, in effect creating a humanized p53 fly set (hereafter, designated HPFS).

Human p53 mutants are not intrinsically stable

Accumulation of mutant p53 is a common hallmark of cancer routinely used to identify tumor tissue(15). To determine whether mutant p53 proteins accumulate in humanized p53 flies, we blotted HPFS ovary lysates with DO-1, a monoclonal hp53 antibody that can detect both wild-type and mutant hp53 protein. Figure 1b shows that in this heterologous context, hp53 variants were expressed at levels comparable to that of the wild-type hp53 lines, consistent with reports showing that hp53 mutant proteins are not intrinsically more stable than wild-type hp53(6, 7, 16).

Human p53 functionally complements the Drosophila counterpart

To determine whether human p53 could functionally replace fly p53 in *Drosophila*, we conducted complementation tests and assessed whether hp53 rescued defective phenotypes seen in Dp53 mutants. Like mammalian systems, flies mutant for p53 are defective for apoptosis provoked by genotoxic stressors such as gamma-irradiation (IR). To determine whether this apoptotic response is rescued in humanized p53 flies, we stained irradiated embryos and wing discs with acridine orange (AO), a dye that detects apoptotic cells *in vivo* (17). Hp53 embryos and wing discs did not exhibit signs of apoptosis under normal conditions but a partial apoptotic response was observed following irradiation (Figure 1c and 1d). We also tested whether hp53 could restore transactivation, using an *in vivo* reporter transgene. To this end, we introduced a previously validated p53 biosensor (p53R-GFPcyt, see Supplementary Figure 1 for more details) into all of the HPFS strains and examined samples post-irradiation (IR). As expected(18, 19), the *Dp53*$^{+}$ rescue fragment restored endogenous (Supplementary Figure 1a) and stimulus-dependent (Figure 1e) biosensor activation in the ovaries of *dp53*$^{-/-}$ female flies as determined by GFP staining. Notably, hp53 also restored activation of the reporter in the ovaries, albeit in a more extensive fashion than *Dp53*$^{+}$ (Figure 1e and Supplementary Figure 1a). All five hp53 mutants, however, were impaired for apoptosis in the wing disc (Supplementary Figure 2) and silent for biosensor activation (Figure 2a and Supplementary Figure 1b), indicating that these mutants are defective for transactivation of target genes, as expected(6, 7). Western blot analysis confirmed absence of biosensor activation by hp53 mutants and also verified that failure to activate the biosensor was not caused by diminished hp53 protein levels, since the mutant and wild-type hp53 lines express hp53 at comparable levels (Figure 2b). To extend these findings, we similarly tested a validated panel of radiation-induced p53-dependent (RIPD) genes previously characterized in the fly embryo(20) (Figure 2c). Hp53 fully rescued *Xrp1* induction and partially rescued *Hid and Eiger*. *Hid* and *Eiger* are both apoptotic activators, suggesting that partial induction of these targets might be sufficient to launch an apoptotic

response in embryos (see Figure 1d). Furthermore, in wild-type hp53 lines, stimulus-dependent transcriptional induction of the biosensor clearly occurred without an accompanied increase in hp53 protein levels (Figure 2b). Hence, contrary to prevailing models(21), stabilization of p53 was not required for stimulus-induced transactivation in this system.

Normal but not mutant hp53 is recruited to SUMO-associated nuclear foci

In order to launch DNA repair and cell death programs, p53 must translocate to the nucleus where it can bind to the promoters of its target genes(21). To address defective nuclear translocation as a possible explanation for the inability of hp53 mutants to activate the p53 biosensor, we stained HPFS ovaries with anti-hp53 and anti-lamin, which stains the nuclear membrane. Hp53 staining was predominantly nuclear in both wild-type and mutant hp53 lines as seen in Supplementary Figure 3a. Hence, we can exclude failure of nuclear transport as a trivial explanation for defective transactivation by hp53 mutants. Interestingly, all HPFS stocks formed small punctae in the cytoplasm of germline cells (quantified in Supplementary Figure 3e). However, we noticed that like its fly counterpart(22), wild-type hp53 assembled large foci, which were particularly conspicuous in differentiating germline cells (Figure 3a), late-stage egg chamber follicle cells (Supplementary Figure 4a) and malpighian tubule (fly kidney) cells (Supplementary Figure 4b). Co-staining with anti-lamin and anti-hp53 revealed that these structures were predominantly nuclear (Supplementary Figure 3a). Strikingly, none of the mutant hp53 proteins formed these prominent nuclear foci in the germline (Figure 3a and Supplementary Figure 3b). We rigorously quantified this using unbiased automated image processing algorithms and determined that all hp53 mutants were clearly distinct from wild type (Figure 3b-dc). As shown previously, this difference is not due to decreased protein levels (Figure 2b) or failure to translocate to the nucleus (Supplementary Figure 3a). These findings uncover intrinsic *in vivo* properties, revealed through subnuclear localization patterns, that distinguish wild-type from mutant hp53 proteins.

To determine whether foci formed by hp53 correspond to the same nuclear bodies that were previously reported for Dp53(22), we costained ovaries from flies expressing both hp53 and endogenous Dp53. As seen in Figure 3d, hp53 and Dp53 foci colocalized frequently in the germline, indicating that both proteins share associations with common nuclear structures. Hp53 and Dp53 foci also colocalized in the nuclei of late-stage egg chamber follicle cells (Supplementary Figure 4a). In colocalization studies, we investigated whether hp53 foci associated with markers for known nuclear structures but found no evidence that hp53 was sequestered to Cajal bodies(23), histone locus bodies(24), insulator bodies(25), centrosomes(26), or the nucleolus (Supplementary Figure 5). Because hp53 is a characteristic component of promyelocytic leukemia protein (PML) nuclear bodies in humans, we also costained with known markers for these bodies. Although PML is found almost exclusively in mammals, *Drosophila* possesses orthologs for other PML body markers, such as SUMO1. The fly genome codes for only one SUMO protein (dSmt3/dSUMO) which can be visualized with anti-dSUMO as discrete regions of staining within nuclei(27). As seen in Figure 3e and Supplementary Figure 4b, we detected considerable colocalization between nuclear hp53 foci and discrete regions of dSUMO staining in the germline and in malpighian tubule cells. These structures might represent invertebrate

counterparts of mammalian PML bodies as suggested by Mauri et al(28) and could provide useful entry points for future functional studies.

Cancer-associated hp53 proteins disrupt wild-type hp53 foci and biosensor activation

It is well known that hp53 mutant proteins exert some of their effects via dominant negative (DN) activity on wild-type hp53(29). To determine whether mutant proteins exert DN activity in our heterologous system, we tested whether these proteins affected foci formation and biosensor activation by hp53. As shown in Figure 4a and 4c, placing hp53 in *trans* to any of the cancer-associated alleles strongly disrupted hp53 foci formation in the female germline. Importantly, this effect strongly correlated with decreased biosensor activity from the wild-type protein (Figure 4b), suggesting that hp53 foci are linked to transcriptional activity in our system. This data recapitulates the dominant negative activity of cancer alleles seen in mammalian models(6, 7) and assigns functional significance to hp53 foci.

In summary, we have validated *Drosophila* as a promising *in vivo* system with which to interrogate p53 alleles and potentially stratify variants seen in the clinic. Our system complements existing models in yeast(30), cultured cells(31) and mice(6, 7). Yeast, however, does not contain a native p53 network nor is it a metazoan with diverse cell types and developmental stages. Relative to mouse models or tissue culture systems, our platform enables sophisticated *in vivo* analyses and genetic screens at unprecedented scales. Since there are no fly orthologs of *Mdm2, p63* or *p73*, our platform can be leveraged to identify intrinsic properties of wild-type and mutant alleles that may be independent of these binding partners. For example, the absence of Mdm2 enabled us to show that stimulus-dependent activation of hp53 can occur without stabilization (Figure 2b), demonstrating that these two processes can be uncoupled. Although such uncoupling is thought to occur in *C. elegans* (32), our model clearly illustrates that stabilization is not needed for activation of the human p53 protein.

We are not the first group to attempt to study human p53 in the *Drosophila* model(33). This is because the p53 regulatory network(18, 34), its consensus binding site(33, 35) and many of its cellular functions(35) are well conserved in the fly. Our approach to engineering this platform is an important advance, as previous attempts to study human p53 in this model relied on ectopic over-expression and caused cell lethality(33). We circumvented this problem by placing hp53 cDNAs under the control of native regulatory elements. Furthermore, outside of the directed base-pair substitutions, all HPFS lines are otherwise identical because the corresponding humanized fragments were placed at the same landing site in the genome. The five cancer-associated mutants tested here failed to activate a p53 biosensor and trigger apoptosis. Furthermore, nuclear foci assembly was drastically impaired in all the hp53 variants despite comparable expression in the nucleus. DNA-damage did not result in any significant changes in foci formation in either wild-type or mutant hp53 (Supplementary Figure 4f). Interestingly, hp53 foci were found to associate with SUMO, a characteristic PML body marker. PML bodies have been associated with various disorders and pathologies including cancer(36) and are associated with some mutant p53 activities(37). Strikingly, presence of cancer-associated hp53 proteins disrupted wild-type hp53 foci and biosensor activity. It will be interesting to further explore whether

disruption of these foci is causally linked to failures in transactivation and apoptotic programming.

The behavior of hp53 mutants in our system is generally consistent with much of what is known about these proteins. Like Li Fraumeni patients and knock-in mice(6), HPFS flies do not show accumulation of hp53 mutant proteins in normal tissues. This supports the current model which proposes additional lesions are required for accumulation of hp53 mutant protein(9). Additionally, our observation that mutants fail to activate the p53 biosensor agrees with studies showing that hp53 mutants lose their ability to transactivate canonical p53 target genes(6, 7, 30). Interestingly, Xu et al(38) and Ano Bom et al(39) reported aggregation behavior of some hp53 mutants but we find no evidence for similar properties here, indicating that aggregation of mutant proteins may involve additional lesions in transformed tissue.

Here, we propose that simple phenotypes such as foci formation could enable methods to eventually stratify understudied variants and identify cancer-relevant alleles. Several studies have described unique properties among hot-spot mutants both in culture and *in vivo* (6–8). In light of this, it is tempting to speculate that differences in the severity of phenotypes (such as foci formation defects) might allow for prognostic stratification of p53 mutations. Future studies will exploit this HPFS platform to examine how properties intrinsically shared by hp53 cancer variants influence cellular behaviors in models of metastasis(40), competition and compensatory proliferation(41).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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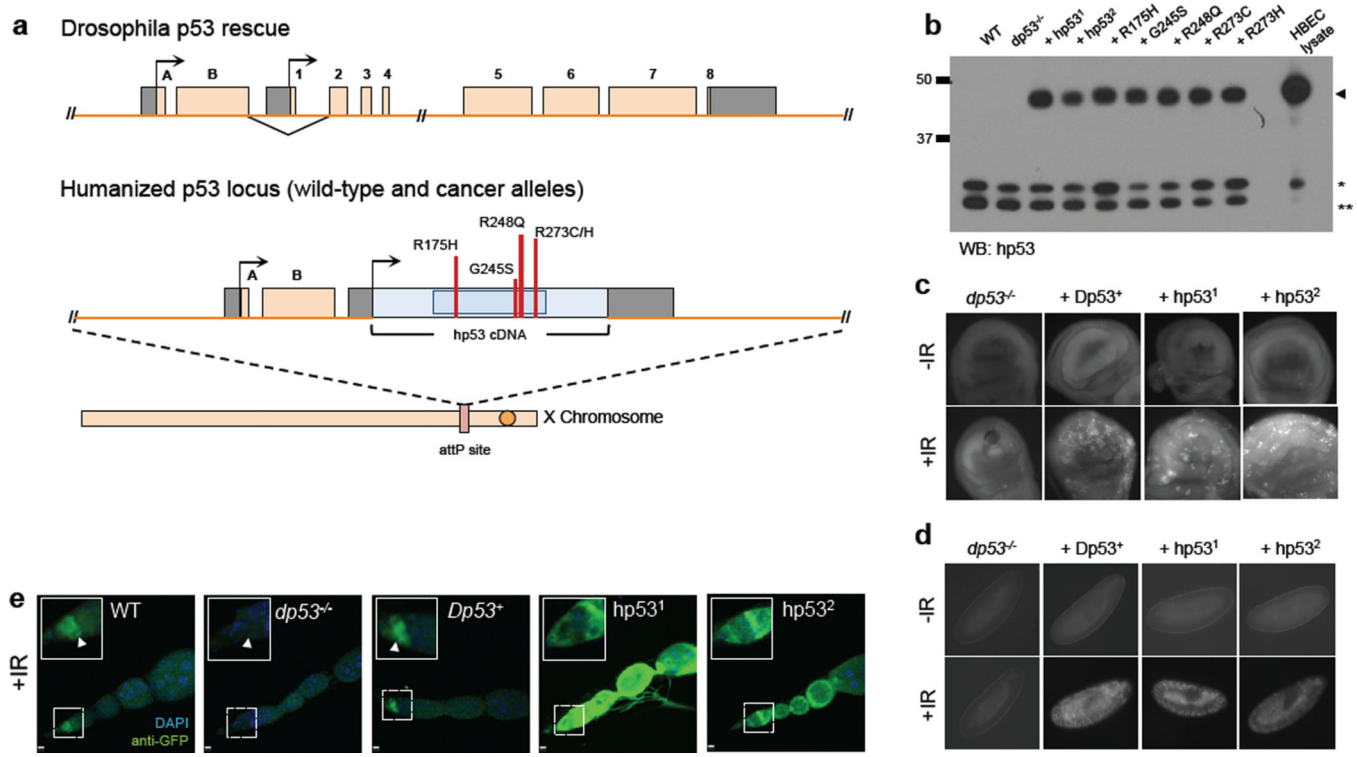

Figure 1. Human p53 functionally complements the Drosophila counterpart

(**a**) The *Dp53*$^{+}$ rescue and the humanized p53 fly set (HPFS) were generated by integrating a BAC containing a 20kb genomic fragment of the *Dp53* locus (CH322-115D03) into an attP landing site on the X chromosome (PBac{y^{+}-attP-9A}VK00006) via phiC31-driven recombination(42). Exons 1-8 of the *Dp53* locus code for the predominant gene product and Exons A&B are alternative exons used in a less abundant isoform. To humanize p53, exons 1-8 of *Dp53* were replaced with wild-type hp53 cDNA (NM_000546.5) via recombineering. To generate hp53 mutant lines, we engineered five of the most prevalent point mutations in human cancer into the cDNA using site-directed mutagenesis. The relative prevalence of the five mutations are indicated by height of the red bars. Orange: *Drosophila* exons, gray: UTRs, blue: human sequence, dark blue: hp53 DNA binding domain. Translation start sites are shown as black arrows. (**b**) Western blot for hp53 levels in HPFS ovary lysates using the hp53 DO-1 monoclonal antibody. Note that wild-type hp53 lines (hp53^{1} or hp53^{2}) and hp53 mutants (R175H, G45S, R248Q, R273C, R273H) express hp53 (black arrow) at similar levels. Human bronchial epithelial cell (HBEC) lysate was included as a positive control for full-length hp53. * and ** are non-specific cross-reacting bands. ** serves as a loading control. (**c-d**) Acridine Orange (AO) staining of wing discs (c) and embryos (d) mock treated (-IR) or irradiated at 40 gray (+IR) as in Akdemir et al(20). Note that *dp53*$^{-/-}$ embryos and wing discs do not elicit an apoptotic response after irradiation. This response is restored by presence of the *Dp53*$^{+}$ rescue fragment and partially restored by hp53. (**e**) Confocal micrographs of immunofluorescence on whole mount ovarioles from HPFS flies carrying a biosensor (*p53R-GFPcyt*) for p53 activity. Flies were irradiated at 40 gray (+IR), their ovaries dissected, fixed, stained, mounted and imaged as in Lu et al(18). An ovary is made up of many ovarioles, which function as egg factories. Insets focus on the germanium, the

region of the ovariole where stem cells (white arrowheads) reside. Note that the *Dp53*$^{+}$ rescue can restore biosensor activation in the stem cells (a, inset) and that hp53 (hp53^{1} or hp53^{2}) activates the biosensor throughout the ovariole. Antibodies: mouse anti-hp53 DO-1 (Santa Cruz, 1:1000), rabbit anti-GFP (Life Technologies, IF:1:500). Genotypes: in (b) WT is *yw* and *dp53*$^{-/-}$ is *dp53*$^{5A\text{-}1\text{-}4/5A\text{-}1\text{-}4}$, in (c-e) all flies are in a *p53R-GFPcyt,dp53*^{NS}*/TM3,Sb* background except for WT which is *w;p53R-GFPcyt*. The *TM3,Sb* balancer chromosome contains a breakpoint in the p53 locus(43) and behaves like a p53 mutant in our hands.

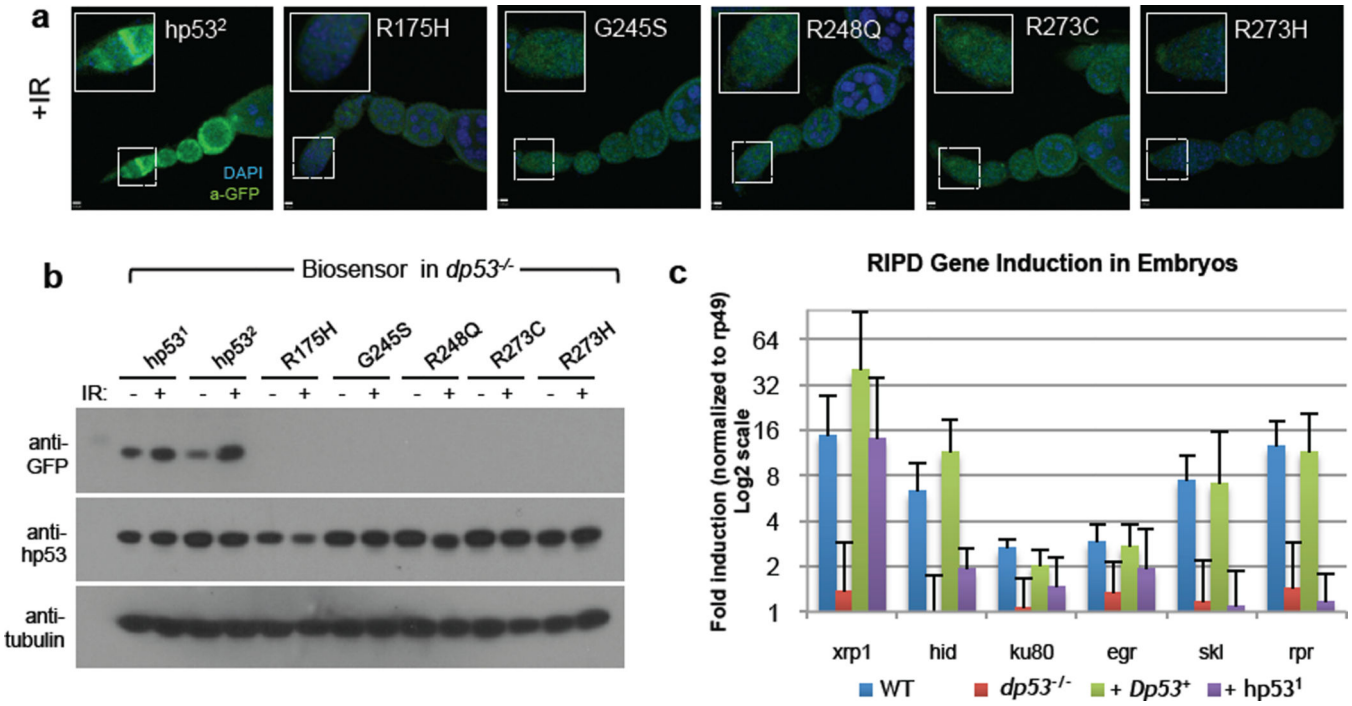

Figure 2. Cancer-associated hp53 alleles are defective for *in vivo* activation of a p53 biosensor

(**a**) Confocal micrographs of immunofluorescence on whole mount ovarioles from HPFS flies carrying a biosensor (p53R-GFPcyt) for p53 activity. Flies were treated and their ovaries stained as in Figure 1E. Note that wild-type ($hp53^1$) but not mutant (R175H, G45S, R248Q, R273C, R273H) hp53 can activate the biosensor throughout the ovariole. (**b**) Western blots of lysates from HPFS ovaries from (a) mock treated (−) or irradiated at 40 gray (+). Note that wild-type hp53 flies show radiation induced upregulation of the p53 biosensor (anti-GFP) without increased stabilization of hp53. Furthermore, all five oncogenic mutants fail to activate the biosensor. (**c**) Embryos of the indicated genotypes were collected for 2 hours, aged for 2.5 hours, mock treated or irradiated at 40 gray, allowed to recover for 2.5 hours and then processed for RNA as in Akdemir et al(20). WT embryos show induction of selected radiation-induced p53-dependent (RIPD) genes(20) after irradiation. This response is lost in $dp53^{-/-}$ embryos and rescued by the $Dp53^{+}$ genomic fragment. Hp53 shows potent rescue of *Xrp1* induction and modest rescue of *hid, ku80* and *egr* induction but fails to rescue *rpr* and *skl* induction. Levels were normalized to rp49. Note log scale to accommodate $Dp53^{+}$ induction of Xrp1. Ovaries were dissected, fixed, stained, mounted and imaged as in Figure 1e. Antibodies: rabbit anti-GFP (Life Technologies, 1:500 (IF), 1:1000 (WB)), mouse anti-hp53 DO-1 (Santa Cruz, 1:1000), mouse anti-tubulin E7 (1:5000 Developmental Studies Hybridoma Bank). Genotypes: HPFS flies in (a) are in a $p53R\text{-}GFPcyt,dp53^{NS}/TM3,Sb$ background and those in (b) in a $p53R\text{-}GFPcyt,dp53^{NS/NS}$ background, and in (c) WT is *yw*, and $dp53^{-/-}$ is $dp53^{5A\text{-}1\text{-}4/5A\text{-}1\text{-}4}$.

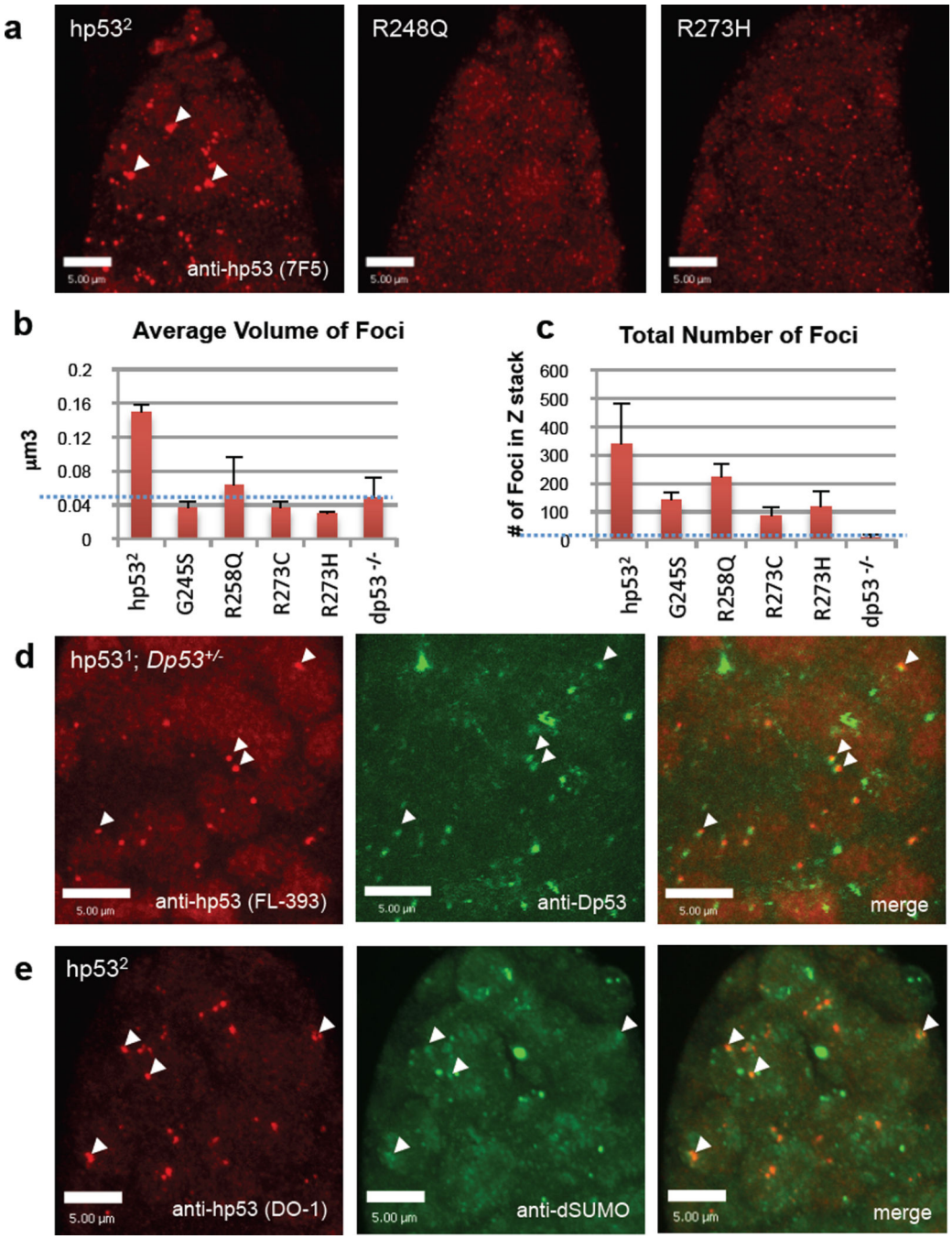

Figure 3. Normal but not mutant hp53 is recruited to SUMO-associated nuclear foci

Confocal micrographs of immunofluorescence on whole mount ovaries. (**a**) Representative germaria of indicated HPFS genotypes stained with anti-hp53 (see Supplementary Figure 3 for the other cancer alleles). White arrowheads indicate representative foci. Note lack of foci in the two representative mutants shown (see Supplementary Figure 3b for the complete HPFS panel). (**b-c**) Quantification of nuclear foci from confocal micrographs of HPFS germaria (see Supplementary Figure 3c) using Imaris (Bitplane).(**b**) Average volume of nuclear foci within a ~50um Z-stack (in um^3). (**c**) Total number of nuclear foci present

within a ~50um Z-stack. Dotted blue line in (b) and (c) represent level of background. Data represents two germaria. Error bars represent SD. (**d**) Germarium showing hp53 (red) and Dp53 (green) colocalization. White arrowheads indicate representative colocalization of hp53 foci and Dp53 foci. (**e**) Germarium showing examples of hp53 (red) and dSUMO (green) colocalization. White arrowheads indicate representative colocalization of hp53 foci and dSUMO staining. Note that with both Dp53 and dSUMO, extensive but not complete colocalization is seen. All images represent collapsed Z-stacks (~50um). Ovaries were dissected, fixed, stained, mounted and imaged as in Figure 1e. Antibodies: rabbit monoclonal anti-hp53 7F5 (Cell Signaling, 1:500), anti-lamin Dm0 (Developmental Studies Hybridoma Bank, 1:100), mouse anti-Dp53 25F4 (Developmental Studies Hybridoma Bank, 1:500), rabbit anti-hp53 FL-393 (Santa Cruz, 1:500), mouse anti-hp53 DO-1 (Santa Cruz, 1:500), rabbit anti-dSUMO (kind gift from Anne Dejean, 1:300). Genotypes: all HPFS flies are in a $dp53^{5A\text{-}1\text{-}4/5A\text{-}1\text{-}4}$ background except for (e), in which $Dp53^{+/-}$ is *+/TM3,Sb*.

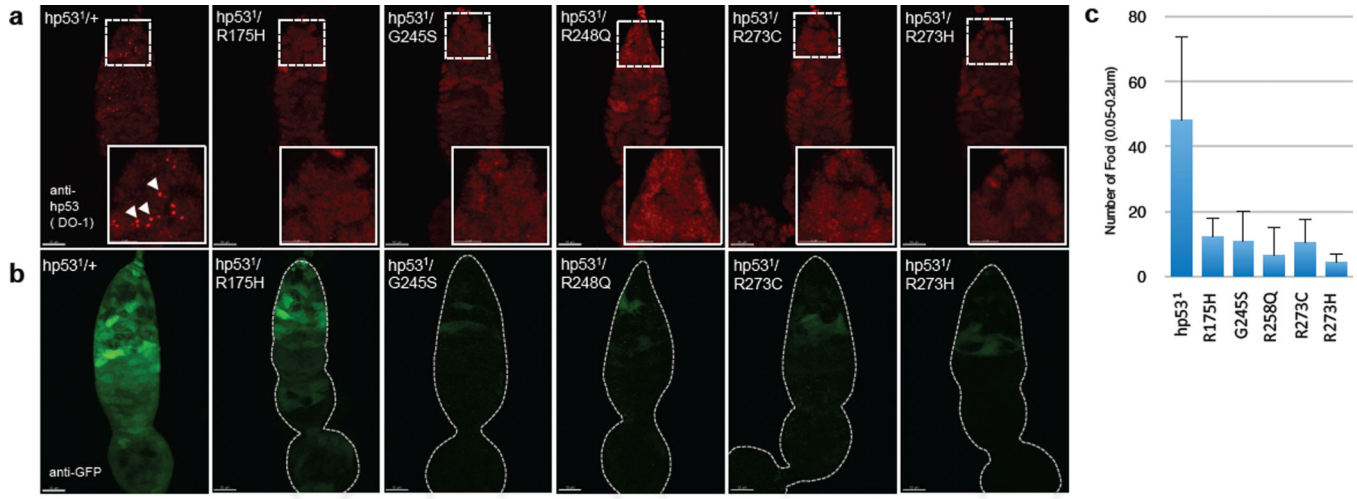

Figure 4. Cancer-associated hp53 proteins disrupt wild-type hp53 foci and biosensor activation

Confocal micrographs of immunofluorescence on whole mount ovarioles from HPFS flies carrying a biosensor (*p53R-GFPcyt*) for p53 activity. (**a**) $Hp53^{1}$ was put in *trans* to either a wild-type chromosome ($hp53^{1}$/+) or to mutant hp53 ($hp53^{1}$/mutant) and then ovarioles were stained for hp53. Note that $hp53^{1}$ can form foci but these do not occur when cancer-associated hp53 proteins are present. White arrowheads indicate representative foci. (**b**) The same ovarioles in (a) stained with anti-GFP to detect biosensor activation. Note that disruption of hp53 foci by hp53 variants results in decreased biosensor activation. (**c**) Quantification of foci present within confocal micrographs in (a). In (c) confocal images were processed using Imaris (Bitplane). Background subtraction was applied, surfaces were created using the hp53 channel to isolate foci between 0.05-0.2 um^{3}, and then values for the total number of foci per Z-stack were plotted. Data represents four germaria. Error bars represent SD. Ovaries were dissected, fixed, stained, mounted and imaged as in Figure 1e. Antibodies: mouse anti-hp53 DO-1 (Santa Cruz, 1:500), rabbit anti-GFP (Life Technologies, 1:500). Genotypes: HPFS flies are in a *p53R-GFPcyt,dp53*$^{NS/5A\text{-}1\text{-}4}$ background. All flies carry two copies of the p53 biosensor.