

The Serine Protease HtrA2 mediates radiation-induced senescence in cancer cells

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1 **Summary**

2 Here the authors identify the Ser protease HtrA2 as a novel mediator of radiation-induced
3 senescence, necessary for sustained proliferation arrest and reorganization of the vimentin
4 filament network.

5

6 **Abstract**

7 Radiation therapy can induce cellular senescence in cancer cells leading to short-term
8 tumor growth arrest, yet increased long-term recurrence. To better understand the molecular
9 mechanisms involved, we developed a model of radiation-induced senescence in cultured cancer
10 cells, which exhibited a typical senescent phenotype, including upregulation of p53 and its target
11 p21, followed by sustained reduction in cellular proliferation, changes in cell size and cytoskeleton
12 organization, and senescence-associated beta-galactosidase activity. A functional siRNA screen
13 using a cell death-related library identified the mitochondrial Ser protease HtrA2 as necessary for
14 senescence development. Mass spectrometry-based proteomic profiling of the senescent cells
15 indicated downregulation of proteins involved in cell cycle progression and DNA repair, and
16 upregulation of proteins associated with malignancy, while irradiation with HtrA2 inhibition
17 upregulated cell proliferation components. In search of direct HtrA2 substrates following radiation,
18 we determined that HtrA2 cleaves the intermediate filament protein vimentin, affecting its
19 cytoplasmic organization. Ectopic expression of active cytosolic HtrA2 resulted in similar changes
20 to vimentin filament assembly. Thus HtrA2, contributes to several hallmarks of senescence and is
21 involved in the cytoskeletal reorganization that accompanies radiation-induced senescence.

22

23 **Introduction**

24 Radiation therapy is often used to induce cytotoxic responses such as apoptosis in tumors,
25 particularly in patients with non-metastatic lung cancer (Heinzerling et al., 2011), but can also lead
26 to survival responses that avoid cell death, such as autophagy and cellular senescence (Patel et al.,
27 2020). Senescent cells are proliferation arrested, and their cytostatic nature precludes further tumor
28 growth. Yet, it is now recognized that at times, the dormant senescent tumor cells can eventually
29 evade growth arrest and regain their proliferative capacity, eventually leading to tumor recurrence
30 (Saleh et al., 2019). In fact, the presence of senescence markers in patients receiving chemo-
31 radiation therapy has been associated with poor prognosis (Rau et al., 2003). On the other hand,
32 combining conventional treatment with anti-senolytic drugs, such as those that inhibit Bcl2 family
33 members, can block tumor progression (Saleh et al., 2020; Shahbandi et al., 2020).

34 Cellular senescence can be a natural consequence of aging and telomere shortening, (i.e.
35 replicative senescence), or of cell stress induced by certain oncogenes, chemotherapeutic drugs or
36 radiation (Hinds and Pietruska, 2017). Proliferation-arrested, senescent cells remain metabolically
37 active and have distinct morphologic and biochemical profiles. Their characteristic features
38 include enlarged area, flattened morphology, a prominent nucleus, increased cytoplasmic
39 granularity, accumulation of heterochromatin foci, and enlargement of the lysosomal fraction
40 including enhanced β -galactosidase activity (known as senescence-associated β -Gal, SA- β -Gal)
41 (Ewald et al., 2010). At the molecular level, senescence results from elevated expression of cyclin-
42 dependent kinase inhibitors such as p21 (*CDKN1A*) and p16 (*CDKN2A*) (Ewald et al., 2010). This
43 leads to suppression of E2F transcription factors that drive expression of genes involved in DNA
44 replication and the G1/S and G2/M transitions (Bringold and Serrano, 2000). DNA damage-
45 induced p53 activation and subsequent increased transcriptional activity is a main regulator of p21
46 expression. As *CDKN2A* is commonly mutated or deleted in cancer, the CDKN2A-RB1 axis is
47 less relevant to the induction of senescence in tumor cells.

48 In order to better understand the contribution of therapy-induced senescence to the
49 suppression of tumor growth on one hand, and to progression and tumor relapse on the other, a
50 more thorough elucidation of its molecular basis is mandated. Although the DNA damage
51 response, p53 up-regulation and cell cycle arrest pathways are well established, very little is known
52 about the molecular mechanisms governing other aspects of senescence, including the

53 characteristic morphologic changes. Here, we addressed this issue in a model of radiation-induced
54 senescence of the non-small cell lung cancer cell line NCI-H460 by applying an siRNA functional
55 screen and characterizing the proteomic profile by mass spectrometry. We used an siRNA sub-
56 library targeting genes from the cell death and autophagy pathways, since senescence and cell
57 death are alternate fates triggered by the same stressors, with molecular cross-talk among the
58 pathways. We thus identified HtrA2, a mitochondrial Ser protease that is released to the cytosol
59 upon cell stress, as an effector driving the re-organization of the vimentin intermediate filament
60 (IF) network, mediating cytoskeletal alterations that occur during cellular senescence.

61

62 **Results**

63 **Characterization of radiation-induced senescence in cancer cells**

64 As a model for therapy-induced senescence, NCI-H460 non-small cell lung cancer cells,
65 which express *TP53* but not *CDKN2A*, were irradiated by clinically relevant doses of X-rays (10
66 Gy). 24 to 48h post-irradiation, morphologic hallmarks of senescence were observed by phase
67 contrast microscopy: most of the irradiated cells were enlarged and flattened, and in contrast to
68 untreated cells, the cells failed to reach confluency (Fig. S1A). This senescent morphology was
69 observed for at least 14d. The number of viable, metabolically active cells, as measured by
70 PrestoBlue assay, started to decline at 24h compared to untreated cells, and continued to decrease
71 with time (Fig. 1A). Quantitation of live cells indicated that the total number of cells dropped
72 dramatically compared to control untreated cells, which continued to proliferate, but did not
73 significantly change over the 72h time-course (Fig. 1B, Fig. S1B). Thus, the large reduction in
74 PrestoBlue values over time most likely does not reflect loss of cells, but rather a decline in their
75 reductive capacity. Staining with calcein/propidium iodide (PI) to measure live/dead cells at 48h
76 indicated a low percent dead cells, which could not account for the large decreases in PrestoBlue
77 values and cell number compared to control (Fig. S1A). Furthermore, no caspase-3 cleavage was
78 evident, and no subG1 DNA was observed, suggesting that apoptosis was not activated in these
79 cells (Fig. S1C,D). Thus, the reduction in cell number compared to control cells most likely reflects
80 a block in proliferation. Cell cycle arrest at the G1 and G2 phases was confirmed by FACS analysis
81 for DNA content: at 24h post-irradiation, cells accumulated at the G1 and G2/M phases at the
82 expense of S phase and did not incorporate bromodeoxyuridine (BrdU), indicative of a block in

83 cell cycle progression (Fig. S1D). Staining for SA- β -Gal and quantification by ImageStreamX
84 flow cytometry indicated an increase in the portion of irradiated cells that were positively stained
85 at 48h and 72h post-irradiation, compared to the control cells (Fig. 1C). Clear differences were
86 also observed when comparing the mean cell areas (Fig. 1D), reflecting the increased cell size
87 following irradiation (Fig. S1E). At the molecular level, protein levels of both p53 and p21
88 increased post-irradiation (Fig. 1E). Altogether, the molecular data, combined with the cell
89 proliferation analysis and observed morphologic changes, indicate that radiation induces sustained
90 senescence in these lung cancer cells. Radiation-induced senescence was also observed in HCT116
91 colon cancer cells, which displayed reduced proliferation, increased cell size, SA- β -Gal
92 accumulation, and induction of p53 and p21 (Fig. S2).

93

94 **siRNA functional screen targeting genes within the programmed cell death pathway** 95 **identifies HtrA2 as a positive mediator of radiation-induced senescence**

96 Having established the senescence phenotype that develops in response to radiation of
97 NCI-H460 and HCT116 cells, we next applied a functional siRNA-based screen for discovering
98 new players that regulate/execute the process. Since a cell's life and death decisions are intricately
99 linked, many points of molecular crosstalk exist within the programmed cell death (PCD) network,
100 and many proteins have dual functions within these various life and death pathways (Bialik et al.,
101 2010). Senescence and cell death are alternative cell fates that are often triggered by the same
102 stressor, and molecular switches can control whether one or the other is activated (Shahbandi et
103 al., 2020; Yosef et al., 2016). Consequently senescence can be considered an arm of the global cell
104 death/viability network. Thus we hypothesized that radiation-induced senescence may be regulated
105 and/or executed by some of the same proteins regulating PCD. In order to identify such dual
106 function players, a small-scale siRNA mediated screen was performed in NCI-H460 cells. The
107 siRNA library, comprising 97 siRNAs targeting apoptosis, programmed necrosis and autophagy
108 genes, and also several known regulators of senescence, was applied to both non-irradiated and
109 irradiated NCI-H460 cells. These were then assayed by CellTiter-Glo (CTG), as a measure of
110 metabolic activity/cell number, to identify PCD genes whose knock-down (KD) affected the
111 senescence response to radiation (Fig. 2A). A reduction in the response following gene KD (i.e.,
112 increased CTG values in irradiated cells) indicates that the corresponding gene functions as a

113 positive mediator of cellular senescence, while enhancement of the response (i.e. decreased CTG
114 values) suggests that either the corresponding gene inhibits senescence, or prevents cell death. Fig.
115 2B shows the fold-change in CTG levels, normalized to non-targeting siRNA control, across the
116 PCD library (see Table S1 for raw and normalized values). The change in CTG was statistically
117 significant for 18 genes (Fig. 2B, red points). The KD of two of these (*BCL2L1* (BCL-X_L) and
118 *CRADD*) resulted in an even greater reduction in CTG activity compared to control (Fig. 2C), the
119 former consistent with previous reports on the senolytic effect of BCL-X_L KD or inhibition (Saleh
120 et al., 2020; Shahbandi et al., 2020; Yosef et al., 2016; Zhu et al., 2015). The KD of the remainder
121 attenuated the response; those with a > 1.5-fold increase in CTG readout are also listed in Fig. 2C.
122 The top hit that passed the FDR correction threshold was *HtrA2* (Fig. 2C). KD of *HtrA2* resulted
123 in a nearly two-fold increase in CTG values following irradiation, normalized to control KD, i.e.
124 the radiation-induced decrease in cell number/metabolic activity was attenuated by depletion of
125 *HtrA2*. This suggests that HtrA2 (also known as Omi), a mitochondrial Ser protease that is released
126 to the cytosol during cell stress (Vande Walle et al., 2008), may be a positive mediator of radiation-
127 induced senescence in NCI-H460 lung cancer cells.

128 Western blot analysis confirmed reduced HtrA2 expression upon introduction of siRNA
129 against *HtrA2* in NCI-H460 cells (Fig. 3A). Consistent with the screen results, microscopy-based
130 examination of irradiated cells indicated that *HtrA2* KD cells were increased in number, reaching
131 near confluency following irradiation, similar to non-irradiated cells (Fig. 3B). The total number
132 of cells (Fig. 3C) and the PrestoBlue values (Fig. 3D) were enhanced by *HtrA2* KD following
133 irradiation, compared to control KD. KD of *HtrA2* also mitigated the radiation-induced increase
134 in SA-β-gal staining compared to irradiated cells transfected with control siRNA (Fig. 3E).
135 Notably, p53 induction was not affected by *HtrA2* depletion (Fig. 3A). In order to evaluate the
136 contribution of HtrA2 to long-term senescence, shRNA against *HtrA2* was used to establish stable
137 KD in NCI-H460 cells (Fig. 3F). Similar to transient KD cells, the stable *HtrA2* KD cell line
138 exhibited reduced SA-β-Gal staining following irradiation, compared to control KD cells, further
139 validating the outcome of *HtrA2* depletion by a different KD strategy targeting different sequences
140 (Fig. S3A,B). Significantly, while irradiated control KD cells maintained reduced PrestoBlue
141 values even at 7d following irradiation, the irradiated *HtrA2* KD cells showed a mitigated
142 senescent response, with increased PrestoBlue values. (Fig. 3F). Finally, to show the relevance of
143 HtrA2 as a regulator of senescence in other cancer cell lines, HCT116 cells were treated with an

144 inhibitor of HtrA2 catalytic activity, Ucf-101, and irradiated. Senescence was measured 48h later
145 by SA- β -gal staining. Significantly, the number of β -gal positive cells decreased in irradiated cells
146 upon HtrA2 inhibition, compared to control treated cells (Fig. 3G). This suggests that HtrA2 is a
147 general positive mediator of radiation-induced senescence in cancer cell lines.

148 **Proteomics profiling of irradiated NCI-H460 cells**

149 In order to broaden our understanding of the signaling pathways governing radiation-
150 induced senescence, to characterize alterations in the proteomic profile induced by radiation, and
151 to study how HtrA2 may be connected to these pathways and changes, mass spectrometry was
152 performed on untreated and irradiated NCI-H460 cells, with or without the HtrA2 inhibitor Ucf-
153 101. A total of ~8000 proteins were identified. There was a close correlation within the 4 biological
154 repeats of each experimental setting, with irradiated samples more closely related to each other,
155 and similarly untreated samples, regardless of drug treatment (Fig. S4A). Quantitative statistical
156 analysis of irradiated vs. control DMSO treated samples revealed differential expression of 317
157 proteins (p and q values < 0.05 , fold change > 2 or < 0.5), the abundance of which increased for
158 170 proteins, and decreased for 147 proteins (Fig. 4A,B and Table S2). As expected, p53 and p53-
159 response genes were among the proteins with increased abundance (24 total, 14.1% of upregulated
160 proteins), including p21, while 107 of the proteins that decreased in abundance (72.8%) were
161 reported targets of p53/p21-mediated DREAM complex transcriptional repression (Engeland,
162 2018; Fischer, 2017). Thus overall, although not excluding translation regulation and post-
163 translation effects, p53-dependent transcriptional regulation potentially accounts for 41% of the
164 up- and down-regulated genes in the dataset, most likely through direct up-regulation of *CDKN1A*.

165 Gene ontology analysis by DAVID software of this set of proteins revealed that, as
166 expected, the top annotation clusters were related to cell proliferation, including mitosis/cell
167 cycle/cell division, DNA replication, cytokinesis, and DNA condensation (e.g. centromere
168 associated proteins, Aurora Kinase B, kinesin family members, condensin complex subunits, DNA
169 polymerase ϵ , DNA topoisomerase 3α), all with reduced abundance, as well as those involved in
170 cell cycle arrest (e.g. p21 (*CDKN1A*) and p27 (*CDKN1B*), both increased in abundance) (Fig.
171 4C). DNA damage/repair cluster was also highly affected by irradiation; most of the proteins in
172 this category were reduced in abundance, and of those, the majority was identified as p53 repressed
173 gene targets (Engeland, 2018; Fischer, 2017) (Fig. S4B). The dataset was also annotated by

174 Ingenuity Canonical Pathway and Disease and Function analyses, which provide information as to
175 the likely activation state of a given pathway or function (i.e. a positive Z-score implies a positive
176 correlation with the activated state). As expected, G1/S and G2/M checkpoints and senescence
177 functions were predicted to be activated, but in contrast, pathways driving cell cycle progression,
178 cell proliferation and cytokinesis functions were predicted to be repressed (Fig. 4D,E). While p53
179 and ATM signaling are likely activated, the BRCA1-related DNA-damage response is likely to be
180 inactivated (Fig. 4D), consistent with the DAVID gene ontology analysis. Interestingly,
181 malignancy functions featured prominently among the biological processes with high positive Z-
182 scores, including pathways such as tumorigenesis, invasion, and metastasis (Fig. 4E). The
183 Ingenuity classifications related to cell death and survival were less informative; cell viability and
184 cell survival functions had both low positive and low negative Z-scores, making these results more
185 difficult to interpret. Of note, the proteins within these categories did not include any of the central
186 regulators or executioners of PCD. Overall, the proteomics analysis of irradiated NCI-H460 cells
187 was consistent with the induction of senescence, but not cell death, with a diminished DNA
188 damage response and surprisingly, up-regulation of proteins associated with malignancy.

189 Comparison of the sets of proteins with altered expression in Ucf-101 treated vs. DMSO
190 treated irradiated cells yielded a smaller group of 51 proteins, 12 of which increased in abundance
191 and 39 decreased (Fig. 4B and Table S3). Ingenuity Disease and Function analysis indicated
192 activation of proliferation functions (Fig. 4F), which contrasted with the predicted inactivation of
193 these pathways by irradiation alone (Fig. 4E). Thus, the effect of HtrA2 inhibition on the proteomic
194 signature of the irradiated cells is consistent with the observed mitigation of the sustained
195 proliferative block in response to radiation shown in Fig. 3. Notably, p53, and p53-target genes
196 that were up-regulated by irradiation showed no significant change upon HtrA2 inhibition.
197 Likewise, the majority of the 107 p53 repression targets that were reduced following irradiation
198 were not affected by the addition of the HtrA2 inhibitor, with the exception of 2 targets whose
199 abundance increased (RAD54B, DHCR7) and 3 others that showed an even greater reduction in
200 the presence of Ucf-101 (CENPH, ELF1, HIRIP3). Thus, consistent with the western blotting
201 showing persistent induction of p53 and p21 following irradiation even upon HtrA2 KD or
202 inhibition (Fig. 3A, Fig. S3C), the MS data suggest a role for HtrA2 downstream to p53 function.

203

204 **HtrA2 cleaves vimentin and affects vimentin assembly and polymerization following**
205 **irradiation**

206 To investigate the p53-independent function of HtrA2 in senescence, we focused on
207 identifying HtrA2 cytosolic substrates, as previous work demonstrated that it is released from the
208 mitochondria into the cytosol following cell stress (Martins, 2002). HtrA2 was shown to cleave
209 the IF vimentin *in vitro*, resulting in the removal of part of its N-terminal head domain, yielding a
210 truncated protein of 49 kDa (Vande Walle et al., 2007). Therefore, vimentin expression was
211 assessed here by western blot to determine if it is cleaved in senescent cells. As shown in Fig.
212 5A,B, a faster migrating form of vimentin was observed following irradiation in NCI-H460 cells,
213 consistent with the proteolytically cleaved form of vimentin (Lucotte et al., 2015). The levels of
214 this smaller form of vimentin was reduced in HtrA2 KD cells (Fig. 5A) and upon treatment of cells
215 with Ucf-101 to inhibit HtrA2 (Fig. 5B). Thus, cleavage of vimentin during radiation-induced
216 senescence requires HtrA2 proteolytic activity.

217 Previous reports documented that the N-terminal head domain of vimentin is essential for
218 vimentin filament assembly (Beuttenmüller et al., 1994; Dave and Bayless, 2014). To determine
219 if vimentin assembly or localization is affected by senescence in an HtrA2 dependent manner,
220 immunostaining for endogenous vimentin was performed in irradiated NCI-H460 cells treated with
221 Ucf-101, which more uniformly achieves HtrA2 inhibition compared to transient siRNA
222 transfection (Fig. 5C). The intensity of vimentin staining was significantly reduced following
223 irradiation. Most importantly, inhibition of HtrA2 activity by Ucf-101 blocked this decline,
224 without affecting the overall shape of the senescent cells (Fig. 5C,D). Notably, no obvious changes
225 were observed in the actin cytoskeletal network upon addition of Ucf-101 within the 48h time
226 frame post-irradiation (Fig. 5C).

227 A more comprehensive analysis of vimentin organization in the irradiated cells revealed
228 prominent, variable changes in vimentin IF assembly and distribution, which were mitigated by
229 Ucf-101. Most of the irradiated cells (69%) exhibited diffuse distribution of vimentin throughout
230 the cytoplasm, without visible filaments (Fig. 6A), whereas a minority of cells (11%) displayed
231 discrete, well organized filaments in either the perinuclear region or uniformly distributed
232 throughout the cytoplasm. A fraction of cells (20%) displayed a combination of diffuse and
233 discrete filamentous organization (Fig. 6A), probably representing a transient intermediate stage

234 in the senescence-related IF disassembly. These changes in vimentin organization were HtrA2
235 dependent, as the addition of Ucf-101 to the irradiated cells significantly reduced the fraction of
236 cells displaying the diffuse pattern (17%), and the fraction of cells exhibiting well-organized
237 discrete filaments reached 66% (Fig. 6B).

238 In many irradiated cells that did not exhibit discrete vimentin filaments, globular dot-like
239 aggregates were detected. These aggregates are presumed to be fragmented vimentin filaments, as
240 described previously (Beuttenmüller et al., 1994). Cells were visually scored for these aggregates
241 as '0' (no visible aggregates), +, ++, and +++ (increasing amounts of vimentin aggregates), as
242 shown in Fig. 6C. Quantitative analysis of these categories indicated that 52% of irradiated DMSO
243 treated cells contained large amounts of vimentin aggregates, with no visible aggregates in only
244 19%. In contrast, only 16% of irradiated Ucf-101 treated cells had large number of aggregates,
245 while 49% did not show any dot-like aggregates (Fig. 6D).

246 To test whether the above mentioned changes in vimentin filament organization is
247 exclusively driven by HtrA2's presence in the cytosol, NCI-H460 cells were transfected with a
248 plasmid driving the expression of a mature, active HtrA2 lacking the mitochondrial targeting signal
249 and tagged at the C-terminus with GFP (HtrA2 Δ 133-GFP) (Fig 7A). Transfection with free GFP
250 was used as a control. To avoid apoptosis due to expression of large quantities of cytosolic HtrA2,
251 the pan-caspase inhibitor Q-VD-OPh was added. As expected, HtrA2 Δ 133-GFP localized to the
252 cytosol in non-irradiated cells (Fig. 7B). The average vimentin staining intensity was significantly
253 lower in the presence of HtrA2 Δ 133-GFP, compared to GFP control (Fig 7B,C). Furthermore, the
254 majority of HtrA2 Δ 133-GFP transfected cells exhibited a diffuse distribution of vimentin filaments
255 (70% vs. 14% of GFP transfected cells), with only 8% exhibiting the organized discrete filaments,
256 compared to 65% of GFP expressing cells (Fig 7B,D). Notably, phalloidin staining of HtrA2 Δ 133-
257 GFP transfected cells indicated that HtrA2 had no obvious effects on the actin cytoskeletal network
258 (Fig 7B). Thus ectopic expression of cytosolic active HtrA2 results in similar changes to vimentin
259 assembly as induced by radiation.

260 In conclusion, following irradiation, concomitant with the acquisition of a senescent state,
261 HtrA2 drives a substantial disruption of the vimentin-based IF network, characterized by decreased
262 amount of visible filaments and prominent aggregates, consistent with filament fragmentation, and
263 decreased overall vimentin staining intensity. Inhibition of HtrA2's catalytic activity attenuated
264 this process, by significantly preventing the changes in the vimentin filament network. Thus HtrA2

265 is responsible for these specific cytoskeletal changes associated with radiation-induced
266 senescence.

267

268 **Discussion**

269 As shown in this study, radiation induces cellular senescence in NCI-H460 lung cancer
270 cells and HCT116 colon cancer cells, as defined by long-term proliferation arrest, changes in cell
271 morphology and size, reduced metabolic activity and enhanced β -gal staining. A functional siRNA
272 screen of PCD proteins aimed to uncover novel regulators of radiation-induced senescence
273 identified HtrA2 as a positive mediator. The contribution of HtrA2 to several aspects of the
274 senescence phenotype, including reduced proliferation and SA- β Gal staining, was confirmed in
275 different cancer cell lines, using several KDs approaches, including an siRNA pool and shRNA
276 targeting different sequences, and the specific HtrA2 inhibitor, Ucf-101. HtrA2 is a Ser protease
277 and chaperone that localizes to the mitochondrial inter-membrane space, from where it maintains
278 mitochondrial homeostasis (Vande Walle et al., 2008). In this capacity, its inactivation is
279 associated with neurodegenerative disease and aging (Martins et al., 2004). During cell stress,
280 however, HtrA2 is released to the cytosol as a result of changes in mitochondria (Vande Walle et
281 al., 2008). Within the cytosol, HtrA2 has been shown to induce apoptosis through multiple
282 pathways, including binding to and cleaving IAPs (Martins, 2002; Yang et al., 2003). In the current
283 study, we show for the first time that in addition to its known role in apoptosis, HtrA2 is involved
284 in the regulation of senescence. As apoptosis is not induced in these cells following radiation;
285 either additional factors counter HtrA2's ability to cleave its apoptotic substrates, or the amounts
286 of HtrA2 released to the cytosol are limiting. In either case, HtrA2 is likely to have alternative
287 targets/substrates that require its protease activity for generating part of the senescence phenotype.

288 HtrA2 suppression had profound effects on the altered IF organization of the senescent
289 cells, providing for the first time, a mechanistic explanation for cytoskeletal changes associated
290 with senescence. HtrA2 has been shown in the past to cleave several cytoskeletal proteins *in vitro*,
291 including actin and vimentin (Vande Walle et al., 2007), and to modulate G/F-actin dynamics in
292 Ras-transformed senescent fibroblasts by directly cleaving β -actin (Yamauchi et al., 2014). HtrA2
293 has also been shown to cleave the type III IF vimentin in stressed neuronal cells (Lucotte et al.,
294 2015). While no major changes in the actin filament network were observed following HtrA2
295 inhibition or overexpression in the current study, HtrA2 proteolytic activity was necessary for

296 vimentin cleavage following radiation in NCI-H460 cells. This cleavage was previously shown to
297 remove the first 40aa of vimentin, resulting in removal of part of the 95aa N-terminal head domain
298 (Vande Walle et al., 2007). Vimentin's central rod region mediates dimerization and alignment of
299 the dimer into an antiparallel, staggered tetramer, and the exposed head domain interacts with
300 various cellular components, such as lipids, plasma membrane, DNA and RNA. It is also the site
301 of various post-translational modifications, including several phosphorylation events, which affect
302 overall filament assembly. Moreover, the most N-terminal half of the head domain is directly
303 necessary for filament assembly of the tetramers (Herrmann and Aebi, 2004). In fact, we show
304 here that filament assembly is disrupted following irradiation, with increased aggregation and
305 diffuse localization. These aggregates most likely correspond to fragmented vimentin filaments,
306 resembling those described in previous work, in which truncated vimentin deleted of the head
307 domain was introduced into vimentin null cells and led to the formation of vimentin aggregates
308 (Beuttenmüller et al., 1994). These changes in vimentin were blocked by inhibition of HtrA2
309 catalytic activity, and conversely, forced expression of a proteolytically mature form of HtrA2 in
310 the cytosol was sufficient to induce these changes in vimentin filament distribution and assembly.
311 Although only a small portion of vimentin is cleaved, the functional effect on filament assembly
312 is pronounced. This may suggest that the truncated form acts as a dominant negative to affect
313 assembly of the full length protein. While a previous report showed that expression of high levels
314 of human vimentin lacking the entire head in mouse fibroblasts did not affect endogenous vimentin
315 organization (Andreoli and Trevor, 1994), other reports are more consistent with the dominant
316 negative hypothesis. For example, some vimentin head mutants and truncations failed to form
317 filaments on their own and disrupted wild type endogenous filaments, resulting in the
318 accumulation of aggregates (Beuttenmüller et al., 1994). Interestingly, a mutant variant of
319 vimentin, found in a patient with a premature aging syndrome, promoted the generation of an N-
320 terminal cleaved form, and faster protein turn-over (Cogne et al., 2020). When expressed in
321 vimentin null cells, the mutant failed to form filaments and instead accumulated in cytosolic
322 aggregates, alone or when co-expressed with wild type vimentin. In addition, the cleaved N-
323 terminus fragment, although not observed on western blots due to its small size, may remain stable
324 and interact with the acidic central rod domain of intact vimentin. In fact, the head domain was
325 shown to prevent assembly of vimentin into filaments and unravel already formed filaments *in*
326 *vitro* (Traub et al., 1992). Altogether, the data suggest that the direct cleavage of vimentin by

327 HtrA2 within the head domain is the main cause for the loss of vimentin filament organization,
328 although indirect effects of HtrA2 that may influence vimentin assembly by other mechanisms are
329 not excluded. In any case, HtrA2's effects on vimentin assembly underlies the major cytoskeletal
330 change that characterizes senescence in our system.

331 Although absent in normal epithelial cells, vimentin expression is up-regulated during
332 oncogenic transformation (Satelli and Li, 2011), and is considered a hallmark of the Epithelial-
333 Mesenchymal Transition (EMT) in motile epithelial cells during embryonic development and in
334 metastatic cancer cells (Lowery et al., 2015). Changes in vimentin have also been associated with
335 senescence, although the underlying mechanisms were not explored. For example, doxorubicin-
336 induced senescence in A549 lung adenocarcinoma cells also led to changes in vimentin filament
337 organization (Litwiniec et al., 2010), and vimentin filament organization differed between aging
338 senescent and young, proliferating fibroblasts (Nishio et al., 2001). Notably, vimentin intermediate
339 filaments are involved not only in defining cell structure and shape, but also in cellular motility,
340 organelle positioning, and signal transduction (Lowery et al., 2015). Moreover, it has been shown
341 to be necessary for proliferation and tumor growth (Virtakoivu et al., 2015). Thus, the
342 HtrA2/vimentin axis may control several distinct events during cellular senescence, although
343 additional as yet unidentified HtrA2 substrates may also link it to other molecular pathways
344 mediating the various senescent hallmarks.

345 To explore such pathways, we conducted a comprehensive mass spectrometric analysis of
346 the proteome content of irradiated cancer cells undergoing senescence in order to derive a specific
347 proteomic signature that defines these senescent cells and to determine how inhibition of HtrA2
348 affects this signature. To the best of our knowledge, this is the first time such a characterization
349 has been reported in the published literature. It would be interesting to compare this proteomic
350 profile to other cell types, both of normal and cancer origin, and under additional scenarios of
351 stress-induced or replicative senescence, to identify underlying commonalities that define
352 senescence. This may facilitate further mechanistic understanding of the cellular senescence
353 signaling pathway, provide additional molecular markers for monitoring of the process, and
354 potential therapeutic targets to prevent senescence. Interestingly, functions connected to hallmarks
355 of malignancy, including metastasis, tumor invasion and tumorigenesis, were predicted to be
356 activated in the irradiated senescent cells by Ingenuity Diseases and Functions analysis. This
357 finding is counter-intuitive, since it contrasts with the reduced proliferation that was both predicted

358 by the proteomics analysis and observed in the senescent population. However, it is in line with
359 recent clinical outcomes of radiation therapy that suggest that in the long-term, senescence is not
360 a beneficial consequence of cancer therapy. As senescent cells can overcome the mitotic block and
361 regain their ability to proliferate, their presence can lead to tumor regression and resistance to
362 cancer therapy (Krtolica et al., 2001; Saleh et al., 2019). In addition, with time senescent cells
363 develop the senescence-associated secretory phenotype (SASP), which can lead to the
364 development of a pro-inflammatory, immunosuppressive microenvironment that favors
365 tumorigenesis (Hinds and Pietruska, 2017). The expression changes in the dataset that were
366 associated with malignancy, seen early after irradiation, suggest that the senescent cells may
367 already be primed in their current benign state towards this eventuality, even prior to SASP
368 induction.

369 A relatively small set of proteins changed in abundance upon HtrA2 inhibition in irradiated
370 cells. Significantly, these included proteins linked to proliferation, which was predicted to be
371 activated by HtrA2 inhibition. This is consistent with the enhanced cell proliferation observed
372 upon HtrA2 depletion and/or inhibition. Interestingly, the senescence-associated proliferation
373 block was mitigated despite the apparent continued functionality of the p53/p21 pathway, which
374 is the main mechanism for cell cycle arrest in these *CDKN2A* mutated cells. Both p53 and p21
375 were upregulated even upon HtrA2 KD and inhibition, and the MS analysis indicated that HtrA2
376 inhibition did not change the abundance of known p53 target genes nor targets of p53/p21
377 DREAM-mediated repression. This suggests that HtrA2's contribution to senescence, including
378 the sustained proliferation arrest, involves a mechanism independent of or downstream to p53 and
379 p21, elucidation of which requires additional investigation.

380 To conclude, we have identified a novel role for the mitochondrial Ser protease HtrA2,
381 previously implicated in apoptosis, in regulating some of the cytoskeletal changes occurring during
382 radiation-induced senescence. HtrA2 thus joins the short list of proteins, including p53 and BclXL
383 (*BCL2L1*), which modulate apoptotic cell death and cellular senescence (Borras et al., 2020;
384 Vicencio et al., 2008). *BCL2L1* also emerged in our screen as a gene whose KD enhanced the
385 radiation-induced decrease in cell viability. This molecular crosstalk emphasizes the intricacy by
386 which cellular life and death decisions are linked and opens new avenues for discovery of the
387 molecular regulation of cellular senescence.

388

389 **Materials and Methods**

390 **Cell culture and reagents**

391 All reagents were purchased from Sigma-Aldrich unless otherwise indicated. NCI-H460
392 and HCT116 cells were purchased from ATCC and routinely tested for mycoplasma. Cells were
393 cultured in RPMI-1640 medium (Biological Industries, Beit Haemek, Israel), supplemented with
394 10% FBS (GibcoBRL), 4 mM glutamine (GibcoBRL), and combined antibiotics (100 µg/ml
395 penicillin, 0.1 mg/ml streptomycin). NCI-H460 shRNA transfected cells were grown in the
396 presence of puromycin (3 µg/ml). Cells were treated with the following reagents: DMSO, TRAIL
397 (100ng/ml, PeproTech), Ucf-101 (20 µmol/L, Merck), Q-VD-OPh (50 µM, Merck). For radiation
398 experiments, NCI-H460 (8×10^5 cells) or HCT116 (4×10^6 cells) cells were irradiated 24h after
399 plating by X-rays at 10Gy or 8Gy, respectively, in a single fraction, using XRAD 320 (Precision
400 X-Ray). Control cells were subjected to mock irradiation. When indicated, Ucf-101 or DMSO
401 were added 3h prior to irradiation.

402 **Cell viability assays**

403 The number of live cells was determined by Countess cell counter (ThermoFisher) or
404 manually by averaging the number of cells in 4 $1.3 \times 1.75 \text{mm}^2$ fields for each 10cm plate under
405 light microscopy. For PrestoBlue assays, PrestoBlue cell viability reagent (Invitrogen, USA) was
406 added to culture medium according to the manufacturer's protocol. Fluorescence was measured
407 with a microplate fluorescence reader (TECAN, Tecan Trading AG, Switzerland). For CTG
408 assays, luminescence based CellTiter-Glo reagent (Promega, Madison, WI, USA) was added to
409 culture medium according to the manufacturer's protocol. Luminescence was read in a microplate
410 luminometer (TECAN). For calcein/PI assays, 1 µM Calcein AM (Life Technologies) and 1.5 µM
411 PI were added to culture medium 24h post-irradiation and live and dead cells were manually
412 counted under light microscopy (Olympus IX73).

413 **DNA constructs and transfection procedures**

414 GFP was expressed from pEGFP plasmid. pcDNA3-delta133Omi-EGFP was a gift from
415 L. Miguel Martins (Addgene plasmid #14124; <http://n2t.net/addgene:14124>;
416 RRID:Addgene_14124) (Martins et al., 2002). For transient transfection of DNA, TransIT-X2
417 reagent (Mirus) was used according to the manufacturer's instructions. For transient siRNA
418 transfections, 25nM siGENOME siRNA pool to HtrA2 (cat# M-006014-04; see Table S4 for
419 targeted sequences of duplexes in pools) or siCONTROL non-targeting siRNA #5 (NT5, cat# D-

420 001210-05-05) was mixed with Dharmafect1 (Dharmacon), and added to 2×10^6 cells 24h after
421 plating. For stable shRNA transfections, NCI-460 cells were infected with lentiviral particles
422 containing pGIPZ vector carrying shRNA targeting HtrA2 (Dharmacon clone ID V3LHS_315866,
423 mature antisense sequence: ATAAGGTCAGTGTTTCTCG), GAPDH shRNA as positive control
424 or non-silencing shRNA as negative control, according to the manufacturer's protocol
425 (Dharmacon, cat# VGH5526). After continuous selection with puromycin, the clone exhibiting the
426 most prominent decreased expression level of HtrA2 was chosen for further analysis.

427 **Immunostaining and Microscopy**

428 24h post-plating in 35mm glass-bottom microwell plates, NCI-H460 cells were irradiated.
429 After 48h, cells were fixed with 4% paraformaldehyde and 0.1% glutaraldehyde, washed and
430 permeabilized by 0.2% Triton-X100 (Merck), followed by overnight incubation with chicken anti-
431 vimentin polyclonal antibody (Abcam, cat# 24525) in blocking solution of 8% BSA, 0.1% Triton-
432 X100 and then Alexa Fluor 647 conjugated secondary anti-chicken IgG antibody (Invitrogen).
433 Cells were then stained with phalloidin-TRITC and DAPI. Images were acquired with an
434 automated inverted microscope (DeltaVision Elite system IX71 with Resolve3D embedded
435 imaging software; Applied Precision/ GE Healthcare, Issaquah, WA) using a 60x/1.42 oil objective
436 (Olympus, Tokyo, Japan). Further image processing was performed using ImageJ (NIH Imaging
437 Software) (Schneider et al., 2012).

438 For brightfield light microscopy imaging, cells were viewed by UPLFLN PH 10x/0.30 and
439 20x/0.50 objectives on an Olympus IX73 fluorescent microscope equipped with a DP73 camera
440 or an Olympus IX71 fluorescent microscope equipped with a DP70 camera. Images were captured
441 with Olympus CellSens software.

442 **SA- β -gal staining and ImageStream X analysis**

443 SA- β -gal staining protocol and ImageStream X analysis were previously described (Biran
444 et al., 2019). Briefly, irradiated cells were fixed in 4% PFA and stained for SA- β -gal and DAPI.
445 Samples were analyzed by Amnis ImageStream X Mark II (Luminex, Austin, Texas), using
446 dedicated image analysis software (IDEAS 6.2). Cells were gated for single cells according to their
447 area (in μm^2) and aspect ratio (the Minor Axis divided by the Major Axis of the best-fit ellipse) of
448 the brightfield image. To eliminate out of focus cells, cells were gated using the Gradient RMS
449 and contrast features. The percentage of cells positively stained for SA- β -gal was determined by
450 the bright-field mean pixel intensity of each cell (lower values corresponds to higher staining).

451 **Cell cycle analysis**

452 24h following irradiation, NCI-H460 cells were pulsed with 10 μ M BrdU for 3h at 37°C,
453 and detached and trypsinized attached cells collected and fixed in 4% PFA. After consecutive
454 incubations in Denaturation Solution (2N HCl/Triton X-100 in PBS) and Neutralization Solution
455 (0.1M Na₂B₄O₇, pH 8.5) cells were incubated with Antibody Solution (PBS, 0.5% Tween 20, 1%
456 BSA, 1 μ g anti-BrdU-FITC conjugated antibody (eBioscience)) and then resuspended in DAPI
457 solution. Samples were analyzed by ImageStream X Mark II as above. At least 2x10⁴ cells were
458 collected from each sample in each biological repeat.

459 **Protein analysis**

460 Cells were lysed in PLB buffer (10mM NaPO₄ pH 7.5, 5mM EDTA, 100mM NaCl, 1%
461 Triton X-100, 1% Na deoxycholate, 0.1% SDS) supplemented with 10 μ l/ml 0.1M PMSF and 1%
462 protease and phosphatase inhibitor cocktails. For vimentin analysis, cell pellets were lysed in 2%
463 SDS, 50mM Tris-HCl, pH 6.8 or SDS sample buffer (62.5mM Tris-HCl, pH 6.8, 2% SDS, 10%
464 glycerol, 0.1M DTT). Proteins were separated by SDS-PAGE and transferred to nitrocellulose
465 membranes, which were incubated with antibodies against vinculin (Sigma, cat# SA-V9131),
466 cleaved caspase-3 (Cell Signaling, cat# cs9664), tubulin (Sigma, cat# T9026), p21 (Santa Cruz,
467 cat# 6246), GAPDH (EMD Millipore, cat# MAB374), HtrA2 (Cell Signaling, cat# cs2176),
468 vimentin (Sigma, cat #V6389), p53 PAb421 antibody (Bartek et al., 1990). Detection was done
469 with either HRP-conjugated goat anti-mouse or anti-rabbit secondary antibodies (Jackson
470 ImmunoResearch), followed by enhanced chemiluminescence (EZ-ECL, Biological Industries
471 Israel Beit-Haemek Ltd.).

472 **siRNA functional screening**

473 A custom made siRNA library targeting PCD genes (Dharmacon, Table S4) was reverse
474 transfected into NCI-H460 cells plated in two 96-well plates. Several of the siRNAs were present
475 in both plates and served as internal standards. Non-targeting siRNA NT5 was used as control.
476 48h after transfection, one set of the siRNA library pair was irradiated, while its pair was left
477 untreated. Cell viability was measured by CellTiter Glo 48h post-irradiation. The experiment was
478 performed in duplicate and repeated 4 times. Each siRNA readout was log transformed, followed
479 by subtraction of the average siNT5 readouts for each plate respectively (irradiated or untreated).
480 The fold-change was obtained by dividing mean values of irradiated by untreated for each siRNA.
481 The statistical difference between irradiated and untreated samples was assessed using a paired T-

482 test, followed by FDR correction (False Discovery Rate, using p.adjust function) comparing four
483 logarithmic values each for untreated cells and irradiated cells. For both tests, a $p < 0.05$ was
484 considered significant. All calculations were performed with R.

485 **Mass spectrometry based proteomic analysis**

486 Mass spectrometry was performed at the G-INCPM unit, Weizmann Institute. Total protein
487 lysates were prepared from non-irradiated and irradiated cells, treated with DMSO or Ucf-101,
488 48h post-irradiation in four biological replicates. Cells were lysed in buffer (5% SDS, 50 mM Tris-
489 HCl pH 7.4) followed by sonication. Samples were first subjected to in-solution tryptic digestion
490 using the S-Trap method (by Protifi). The resulting peptides were fractionated offline using high
491 pH reversed phase chromatography, followed by online nanoflow liquid chromatography
492 (nanoAcquity) coupled to high resolution, high mass accuracy mass spectrometry (Thermo Q-
493 Exactive HF). Samples from each fraction were analyzed on the instrument separately, and within
494 each fraction, in a random order in discovery mode. Data processing was performed using
495 MaxQuant v1.6.6.0. The data was searched with the Andromeda search engine against the human
496 proteome database appended with common lab protein contaminants and the following
497 modifications: fixed modification (cysteine carbamidomethylation), variable modifications
498 (methionine oxidation), and protein N-terminal acetylation. The quantitative comparisons were
499 calculated using Perseus v1.6.0.7. Decoy hits were filtered out, and only proteins that were
500 detected in at least two replicates of at least one experimental group were kept. A difference ratio
501 for each comparison was calculated based on the average measured protein intensity for each
502 experimental group. A difference in protein level was considered significant if all the following
503 conditions were fulfilled: more than one peptide per protein was identified, the difference ratio
504 was > 2 or < 0.5 , and a p -value lower than 0.05 was calculated. Gene Ontology analysis of mass
505 spec results was done using DAVID v6.8 functional annotation analysis (Huang et al., 2009a;
506 Huang et al., 2009b) or IPA (QIAGEN Inc.,
507 <https://www.qiagenbioinformatics.com/products/ingenuitypathway-analysis>) (Krämer et al.,
508 2014). Top canonical pathways were defined as those with $(-\log(p\text{-value})) > 3$.

509 **Statistical analysis**

510 Statistical analyses were performed using Excel software or R. Values of $p < 0.05$ were
511 considered significant. Specific tests applied to each set of experiments are described in figure
512 legends. Sample variance equivalence was determined by F-test, where appropriate.

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515 insightful discussions.

516 **Competing interests**

517 No competing interests declared.

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520 and from the Minerva Center, Weizmann Institute on "Aging, from Physical Materials to Human
521 Tissues" to BG.

522 **Data Availability**

523 The mass spectrometry proteomic datasets produced in this study are available in the
524 PRIDE PXD020972 repository (<http://www.ebi.ac.uk/pride/archive/projects/PXD020972>)
525 **Username:** reviewer49926@ebi.ac.uk; **Password:** GkCQMcNI.

526

527 **List of Abbreviations**

528 **BrdU**, bromodeoxyuridine

529 **CTG**, CellTiter-Glo

530 **IF**, intermediate filament

531 **KD**, knock-down

532 **PCD**, programmed cell death

533 **PI**, propidium iodide

534 **SA- β -Gal**, senescence-associated β -galactosidase activity

535 **SASP**, senescence-associated secretory phenotype

536

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654

655 **Figure legends**

656 **Fig. 1. Radiation induces senescence in NCI-H460 lung cancer cells. A.** Cells were irradiated
657 and cell number/metabolic was measured by PrestoBlue assay at the indicated times post-
658 treatment. Values were normalized to 0h. Shown is the mean±SD of 3 (24h, 48h) or 4 (72h, 96h)
659 biological repeats per time-point. Statistical significance was determined by Student's two tailed
660 T-test, *** $p = 0.000497$, $p = 0.000574$, $p = 0.000256$, respectively for 48h, 72h, 96h. **B.** Cells
661 were irradiated and live cells counted after the indicated times. Shown is the mean±SD of 4-6
662 individual counts from a representative experiment. Statistical significance was determined by
663 Student's two-tailed T-test, ** $p = 0.00883$, *** $p = 5.63 \times 10^{-10}$. An additional biological repeat of
664 this experiment can be found in Fig. S1B. **C,D.** 48h or 72h post-irradiation, cells were stained for
665 SA-β-gal and DAPI, followed by ImageStream X analysis. At least 2.9×10^4 cells were collected
666 from each sample. Representative distributions indicating intensity of SA-β-Gal staining (C) and
667 cell size (D) are shown. Graph on right in C shows the percentage of positively stained cells, as
668 mean±SD of 5 (48h) or 4 (72h) separate experiments. Statistical significance was determined by
669 two tailed Student's T-test, * $p=0.00685$, ** $p=0.00246$. Graph in D shows the mean cell area±SD
670 of 5 (48h) or 4 (72h) separate experiments. Statistical significance was determined by two tailed
671 Student's T-test, * $p=0.0164$, ** $p = 0.00906$. **E.** Western blots of lysates from cells 48h or 72h
672 post-irradiation.

673 **Fig. 2. Functional siRNA screen for regulators of radiation-induced senescence. A.** Schematic
674 of screen using a PCD siRNA library to identify gene KDs that either enhance or attenuate the
675 cellular senescent response to radiation, assessed by CTG to quantitate changes in metabolic

676 activity/cell number. **B.** Distribution of senescence response of siRNA KDs across the library. For
677 each siRNA, the ratio of CTG values for irradiated vs. untreated cells was calculated. Normalized
678 ratios are plotted for each siRNA, with control non-targeting siRNA values set as 1. siRNAs are
679 distributed along the X-axis in increasing order of the normalized ratios; some were assayed twice
680 as internal plate controls and both outcomes are plotted. Red points indicate siRNAs with
681 statistically significant fold change, $p < 0.05$. **C.** List of genes showing a significant fold-change in
682 the normalized CTG values. Statistical parameters are indicated, significance was determined by
683 paired T-test. For proteins with increased ratio, only those with a fold-change > 1.5 are listed.

684 **Fig. 3. KD or inhibition of HtrA2 blocks radiation-induced senescence in NCI-H460 and**
685 **HCT116 cancer cells. A.** NCI-H460 cells expressing siRNA to HtrA2 or control siRNA were
686 irradiated and western blotted 48h later with antibodies to HtrA2 and p53. Vinculin was used as a
687 loading control. **B.** Light microscopic images of control and HtrA2 KD cells 48h post-irradiation.
688 Bar, 200 μ . **C.** Cells expressing HtrA2 or control siRNAs were irradiated and the total number of
689 viable cells counted 48h later. Shown is mean \pm SD of 3 biological repeats, statistical significance
690 determined by two tailed T-test, * $p = 0.0491$. **D.** Fold-change in PrestoBlue values (irradiated/non
691 irradiated) 48h post-treatment upon KD of HtrA2 by siRNA, with siNT values normalized to 1.
692 Shown is mean \pm SD of 3 biological repeats, statistical significance determined two tailed T-test, *
693 $p = 0.0235$. **E.** siHtrA2 or control NT cells were irradiated and stained for SA- β -gal and DAPI,
694 followed by ImageStream X analysis after 72h. At least 1.3×10^4 cells were collected from each
695 sample. A representative distribution is shown, and graph represents mean \pm SD of 3 experiments.
696 Statistical significance was determined by Student's two tailed T-test, ** $p = 0.00743$. **F.** NCI-
697 H460 cells stably expressing shRNA to HtrA2 or control non-targeting shRNA were irradiated and
698 7d later, subjected to western blotting to confirm KD and PrestoBlue assay to measure viable cell
699 number/metabolic activity. Graph shows mean fold-change (irradiated values divided by control
700 values) \pm SD of 4 technical repeats from 1 of 3 representative experiments, normalized to NT
701 control KD, which was set as 1. Statistical significance was determined by Student's two tailed T-
702 test, *** $p = 0.000889$. **G.** HCT116 cells treated with DMSO or Ucf-101 were irradiated and
703 stained for SA- β -gal and DAPI after 48h. At least 2.6×10^4 cells were collected from each sample
704 and analyzed by ImageStream X. A representative distribution of SA- β -gal positive cells is
705 depicted, and graph represents mean \pm SD of 3 experiments. Statistical significance was determined
706 by Student's two tailed T-test, * $p = 0.0336$.

707 **Fig. 4. Mass spectrometry analysis of irradiated vs. control non-irradiated cells, and upon**
708 **HtrA2 inhibition. A,B.** Volcano plots (left) based on protein LFQ-MS intensity values.
709 Highlighted data points indicate significantly higher (purple) or lower (turquoise) abundance
710 proteins in irradiated vs control cells (A) and in DMSO vs Ucf-101 treated irradiated cells (B), as
711 summarized in pie charts at right. **C.** Gene ontology annotation cluster analysis by DAVID of
712 proteins with altered abundance upon irradiation; top 5 clusters are indicated. **D.** Ingenuity
713 Pathway Analysis showing the top canonical pathways, listed in descending order, of the dataset
714 of proteins with altered abundance following irradiation. Frequency is defined as the number of
715 proteins in dataset/total number of proteins in the given pathway. A positive Z-score indicates
716 likely pathway activation. * Z-score undetermined. **E,F.** Ingenuity Diseases and Functions analysis
717 of the dataset of proteins with altered abundance following irradiation (E) and irradiation with Ucf-
718 101 treatment (F). Highly significant ($p < 10^{-5}$) pathways are shown, grouped by common category.
719 Values on the right refer to the number of proteins in dataset within the given pathway. *p*-values
720 are shown in F.

721 **Fig. 5. Radiation-induced cleavage of vimentin and decreases in vimentin staining intensity**
722 **require HtrA2 proteolytic activity. A., B.** NCI-H460 cells expressing HtrA2 or control siRNA
723 (A) or treated with either Ucf-101 or DMSO as control (B) were irradiated and western blotted
724 48h later with antibodies to HtrA2 or vimentin. Asterisks indicate vimentin cleavage products.
725 Vinculin was used as a loading control **C.** Cells treated with either DMSO or Ucf-101 were
726 irradiated and 48h later immunostained for vimentin. Phalloidin (green) and DAPI (blue) staining
727 was used to detect actin and nuclei, respectively, and are shown in merged images at bottom. Note
728 that all images are the same magnification, bar 10 μ . **D.** Box plot showing the range of vimentin
729 staining intensity measured according to the average pixel intensities inside the contours of each
730 cell. Black lines represent median values, colored regions represent the 25th-75th percentiles, and
731 dots represent potential outliers. Statistical significance was determined by Two-sample
732 Kolmogorov-Smirnov test for two biological repetitions, $p < 2.2e^{-16}$.

733 **Fig. 6. Inhibition of HtrA2 proteolytic activity reduces vimentin filament fragmentation and**
734 **reorganization following radiation. A.,B.** NCI-H460 cells were irradiated and 48h later stained
735 for vimentin (red), actin (phalloidin, green) and nuclei (DAPI, blue). Representative images of
736 vimentin stained filaments depicting each type of organization are shown in A. Arrows indicate

737 cells displaying the relevant category, arrowheads, vimentin aggregates. Brightness and contrast
738 were adjusted when needed across an image to improve visibility of specific structures. Bar, 10 μ .
739 Quantities of each category in DMSO or Ucf-101 treated cells are depicted in B. Approximately
740 200 cells were evaluated in each of two biological experiments. Statistical significance was
741 determined by Chi-square test, **** $p < 0.00001$. Cytosolic and perinuclear localized discrete
742 filaments were considered as one category. **C., D.** Same as A and B, but representing degree of
743 filament fragmentation.

744 **Fig. 7. Expression of cytosolic HtrA2 induces changes in vimentin filament organization. A.**
745 Schematic representation of full length HtrA2 protein, and the cytosolic HtrA2 Δ 133-GFP
746 construct. MLS, mitochondrial localization signal, TM, transmembrane segment, IBM, IAP
747 binding motif, PDZ, C-terminal PDZ domain. **B.-D.** Cells treated with Q-VD-OPh were
748 transfected with GFP or HtrA2 Δ 133-GFP. 48h later, cells were stained for vimentin, actin
749 (Phalloidin, green) and nuclei (DAPI, blue). **B.** Representative images of vimentin staining and
750 merged images. Arrows indicate transfected cells. Bar, 10 μ . **C.** Box plot showing the range of
751 vimentin staining intensity measured according to the average pixel intensities inside the contours
752 of each cell. Black lines represent median values, colored regions represent the 25th-75th
753 percentiles, and dots represent potential outliers. Statistical significance was determined by Two-
754 sample Kolmogorov-Smirnov test for two biological repetitions, $p = 1.184e^{-06}$. **D.** Quantification
755 of vimentin filament organization patterns. Statistical significance was determined by Chi-square
756 test for two biological repetitions, **** $p < 0.00001$.

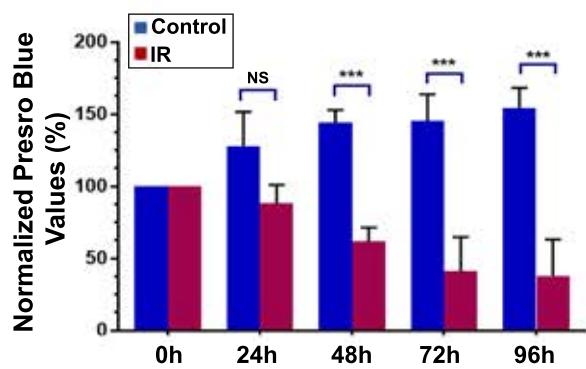
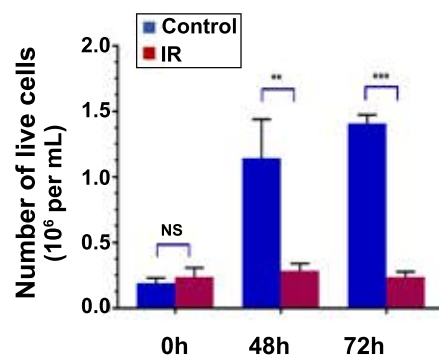
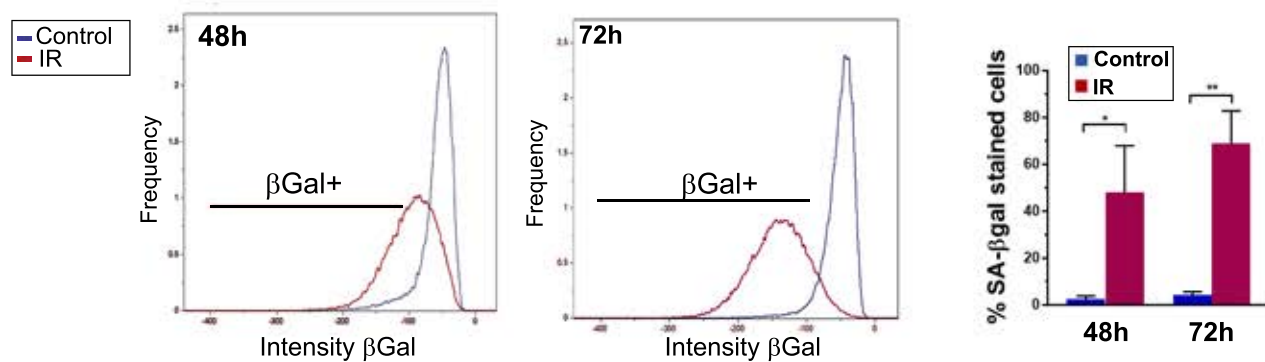
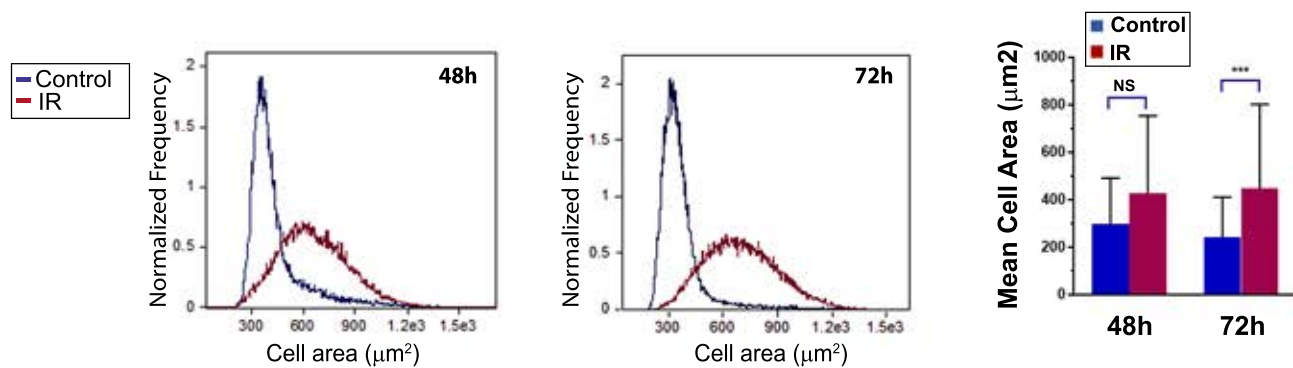
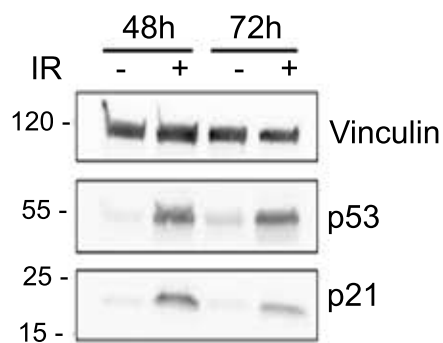
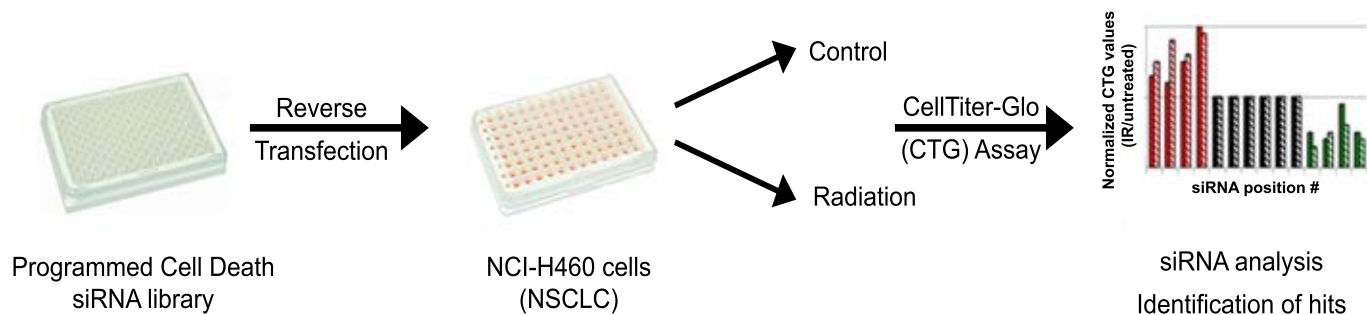
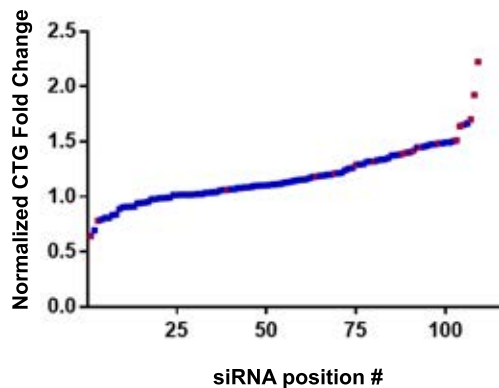
Figure 1**A.****B.****C.****D.****E.**

Figure 2

A.



B.



C.

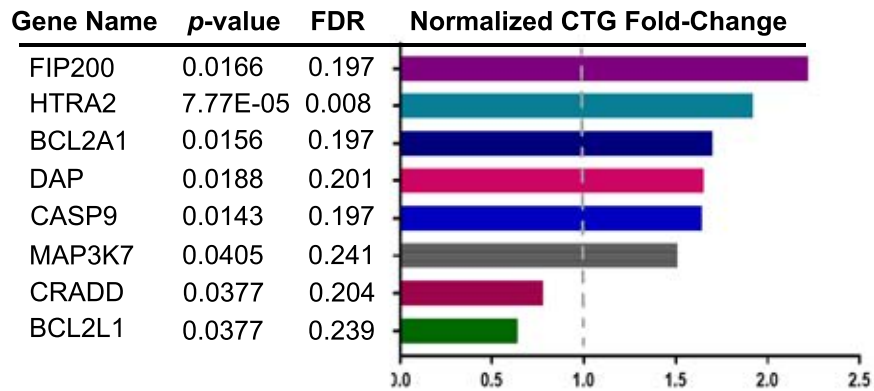
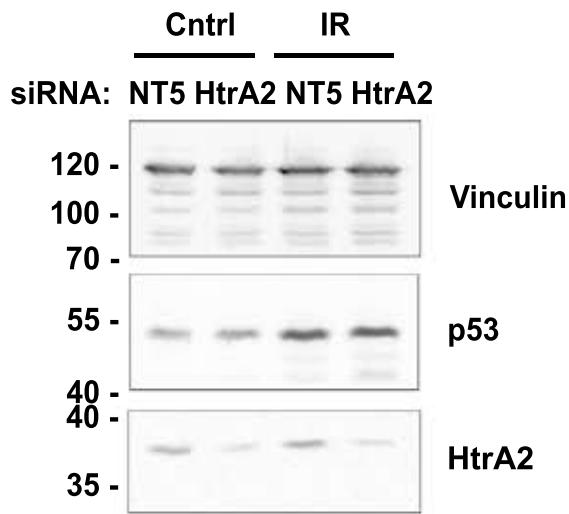
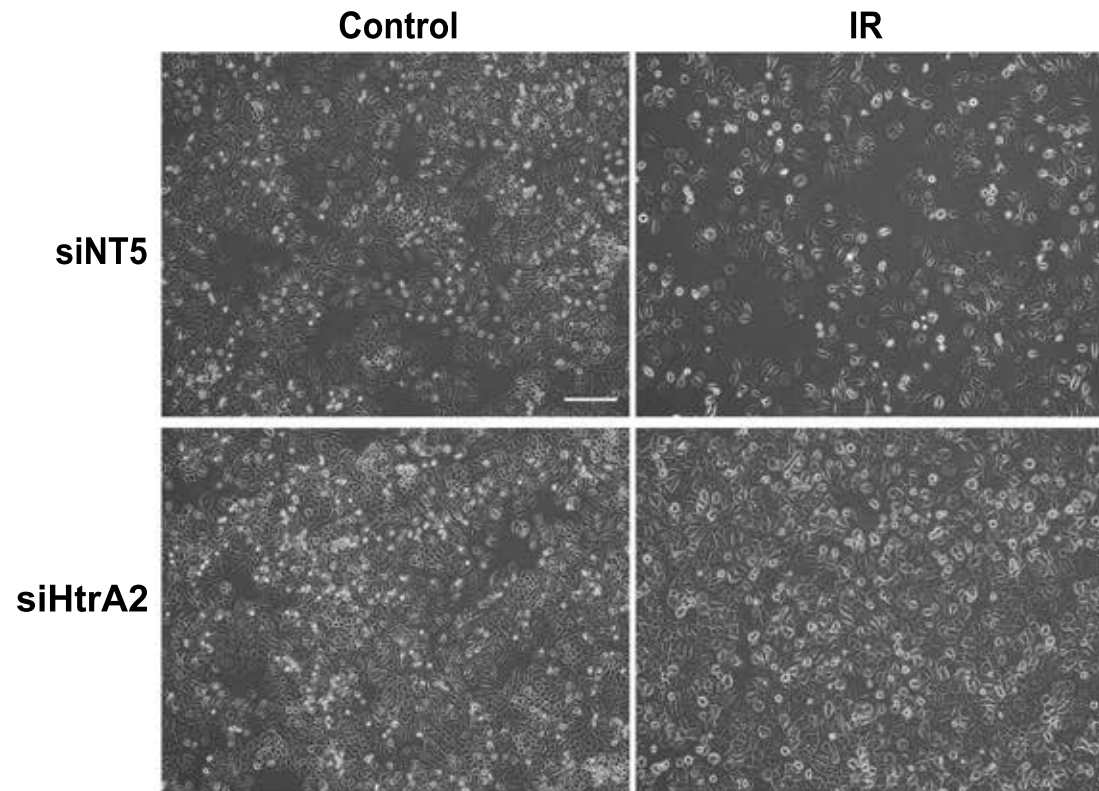


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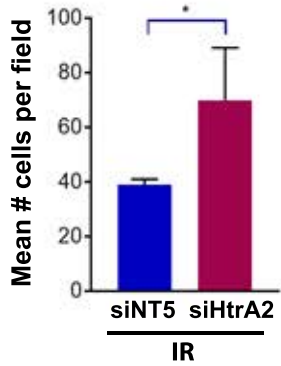
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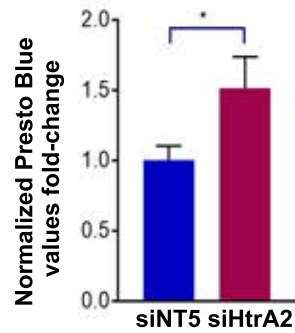
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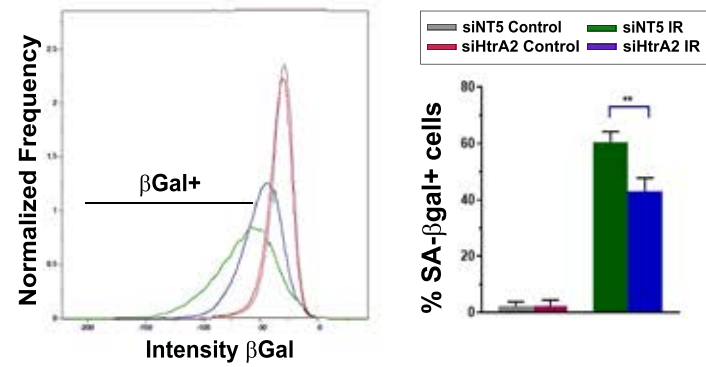
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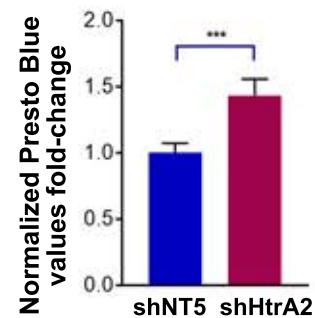
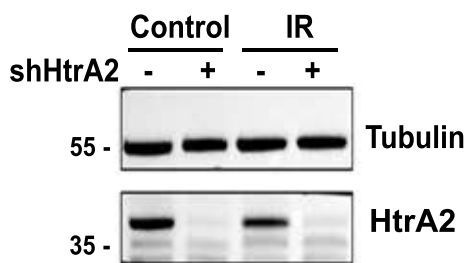
D.



E.



F.



G.

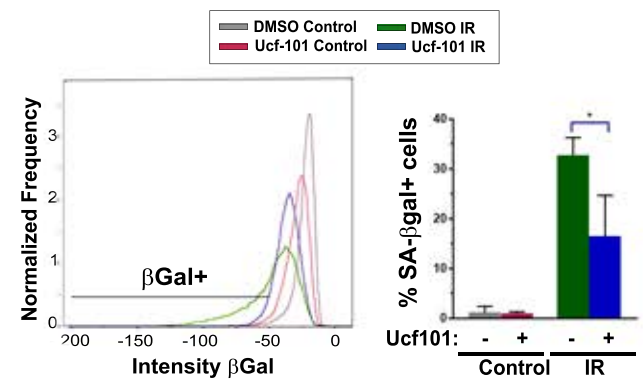
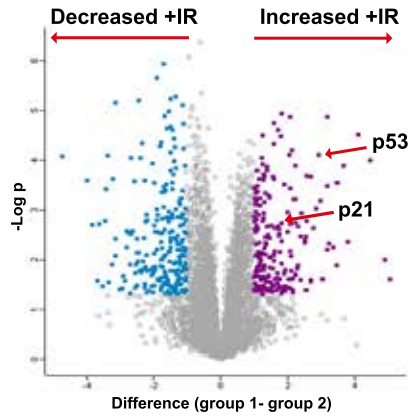
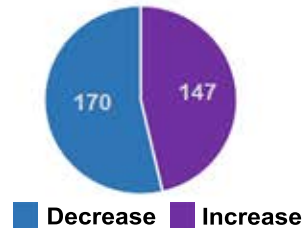


Figure 4

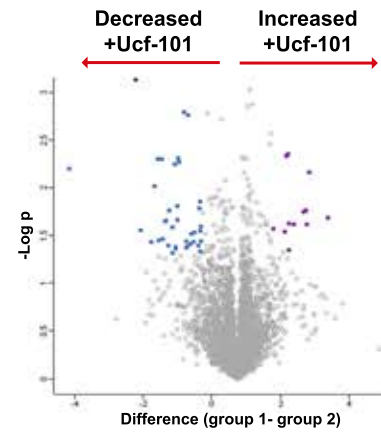
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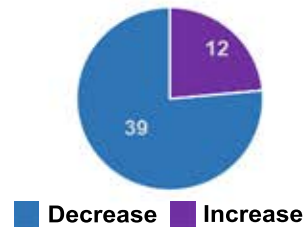
IR vs Control



B.



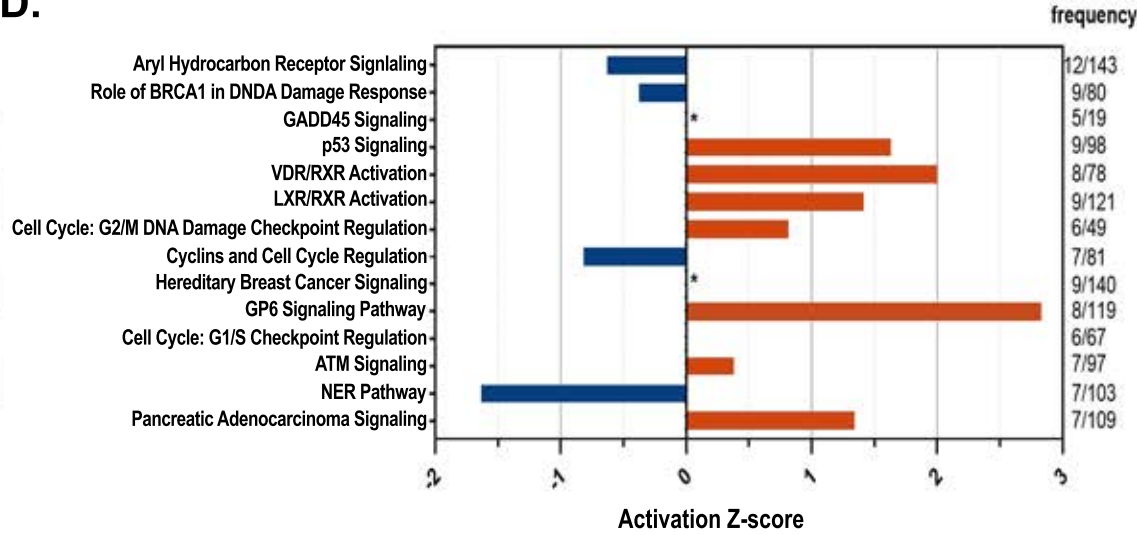
IR Ucf-101 vs DMSO



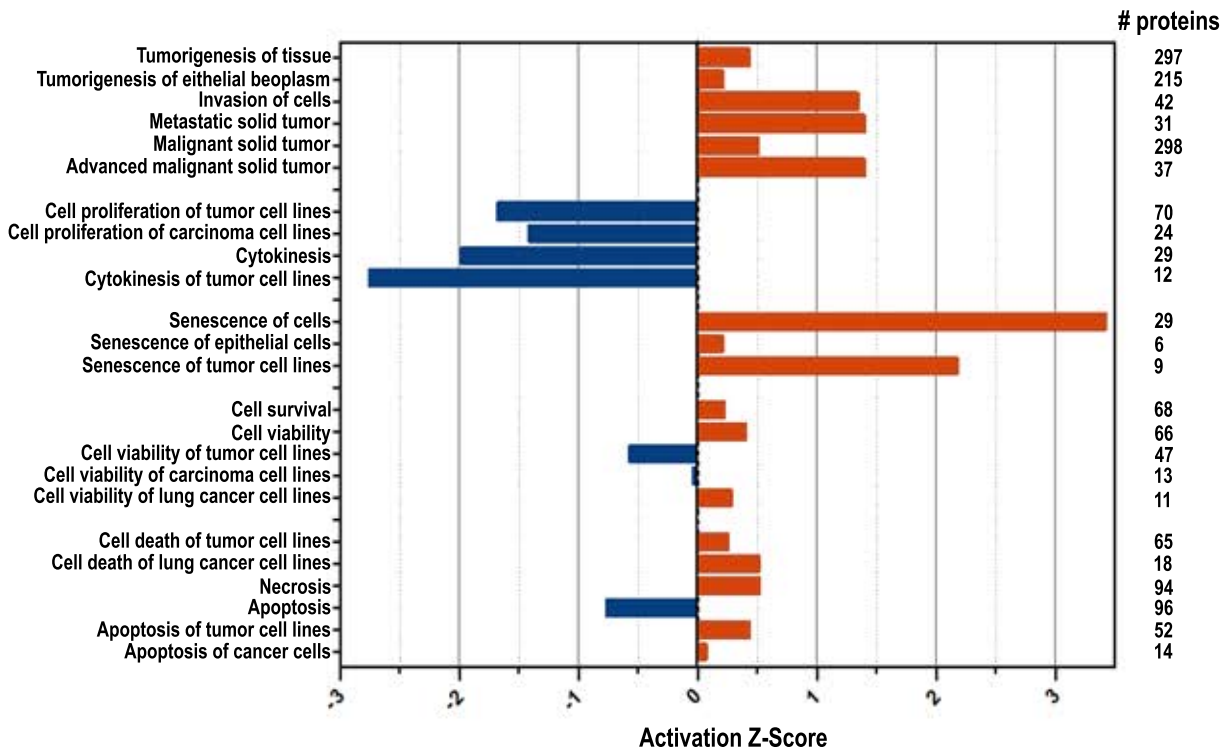
C.

Annotation Cluster	Enrichment Score
Mitosis/Cell Cycle	17.97
Chromosome	11.20
DNA Damage/Repair	7.06
DNA replication	5.54
Cytokinesis	5.02

D.



E.



F.

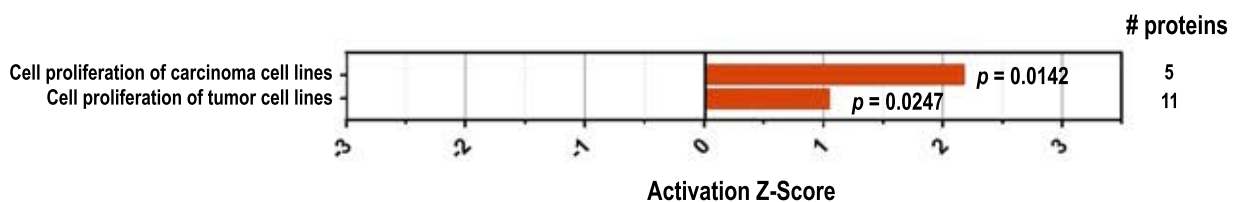
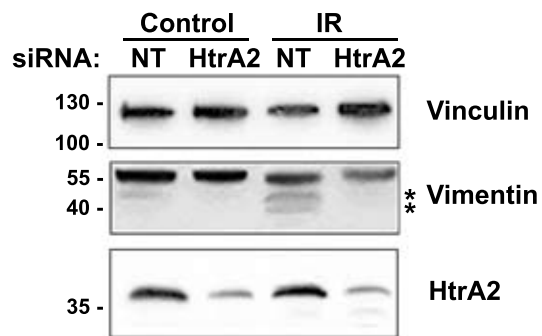
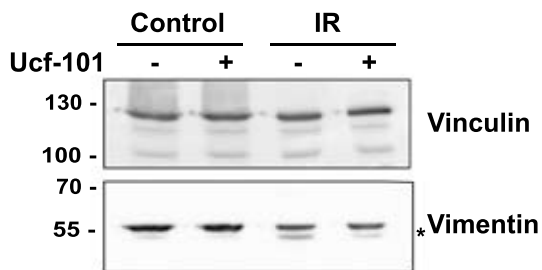


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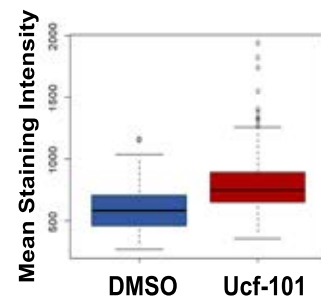
A.



B.



D.



C.

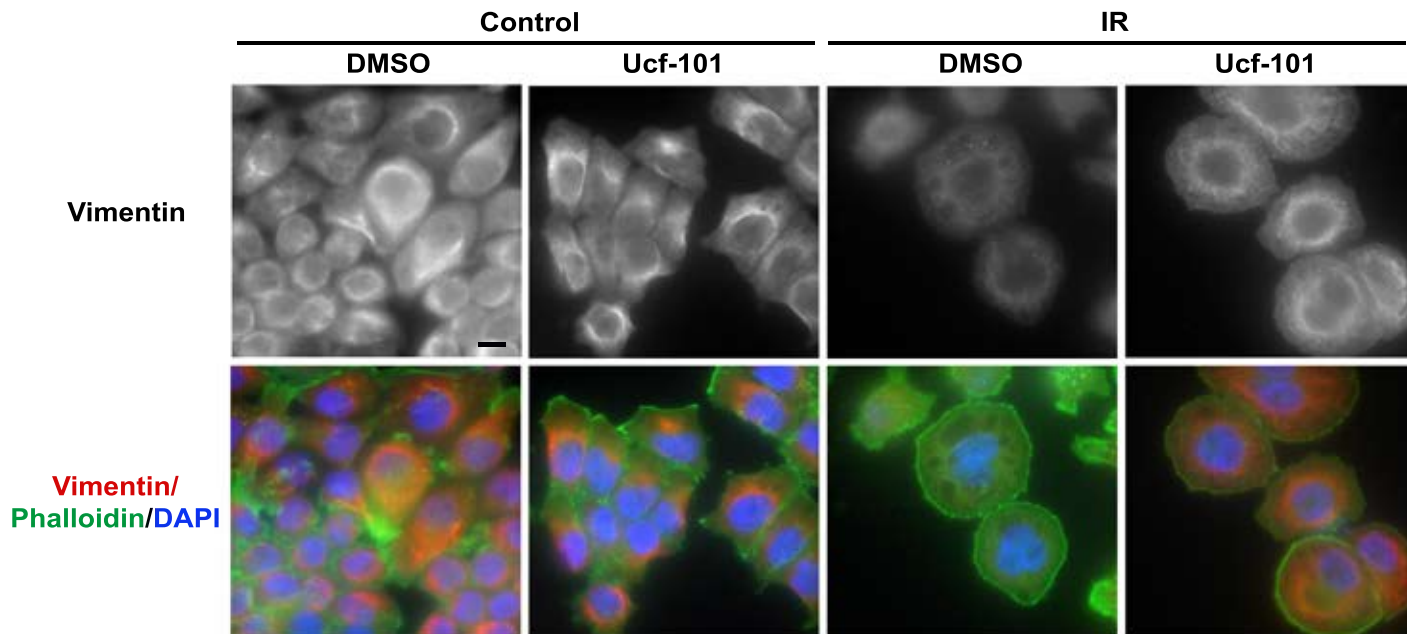
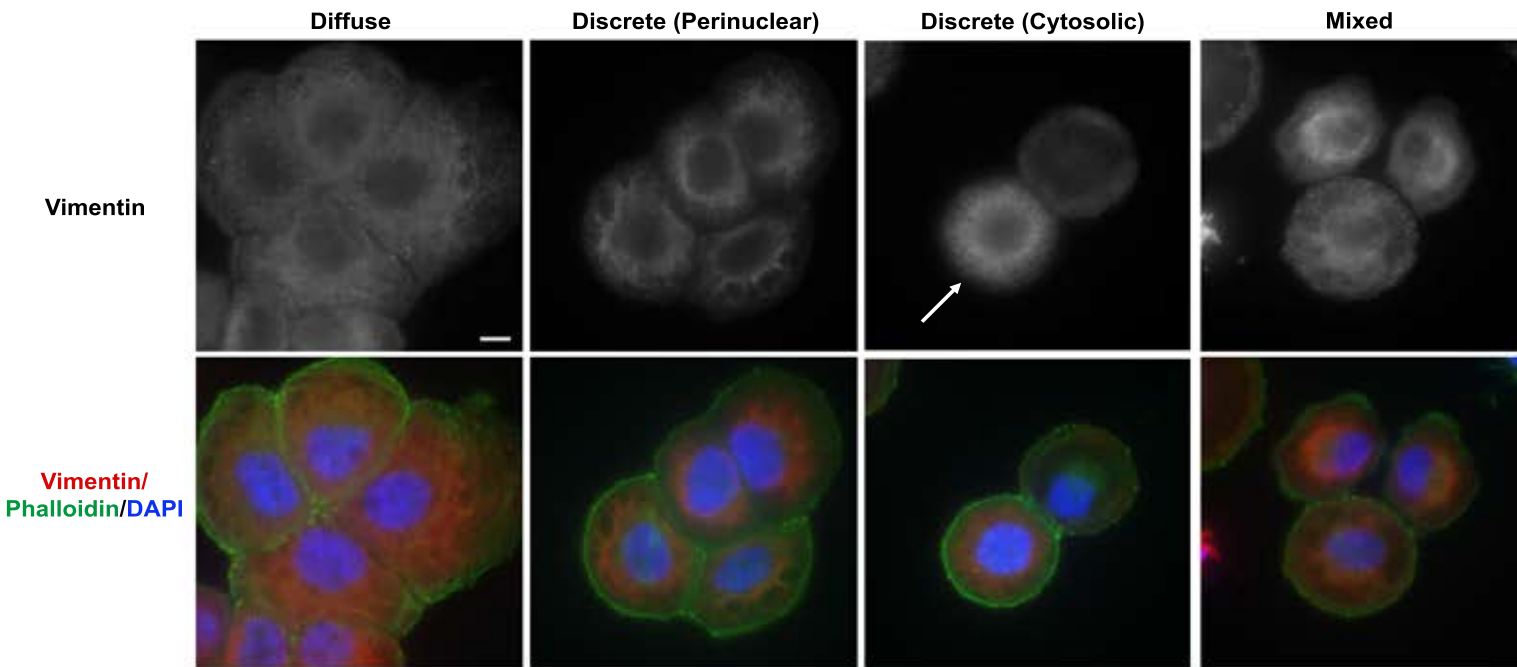


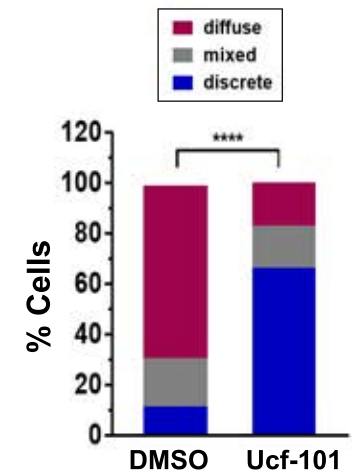
Figure 6

A.

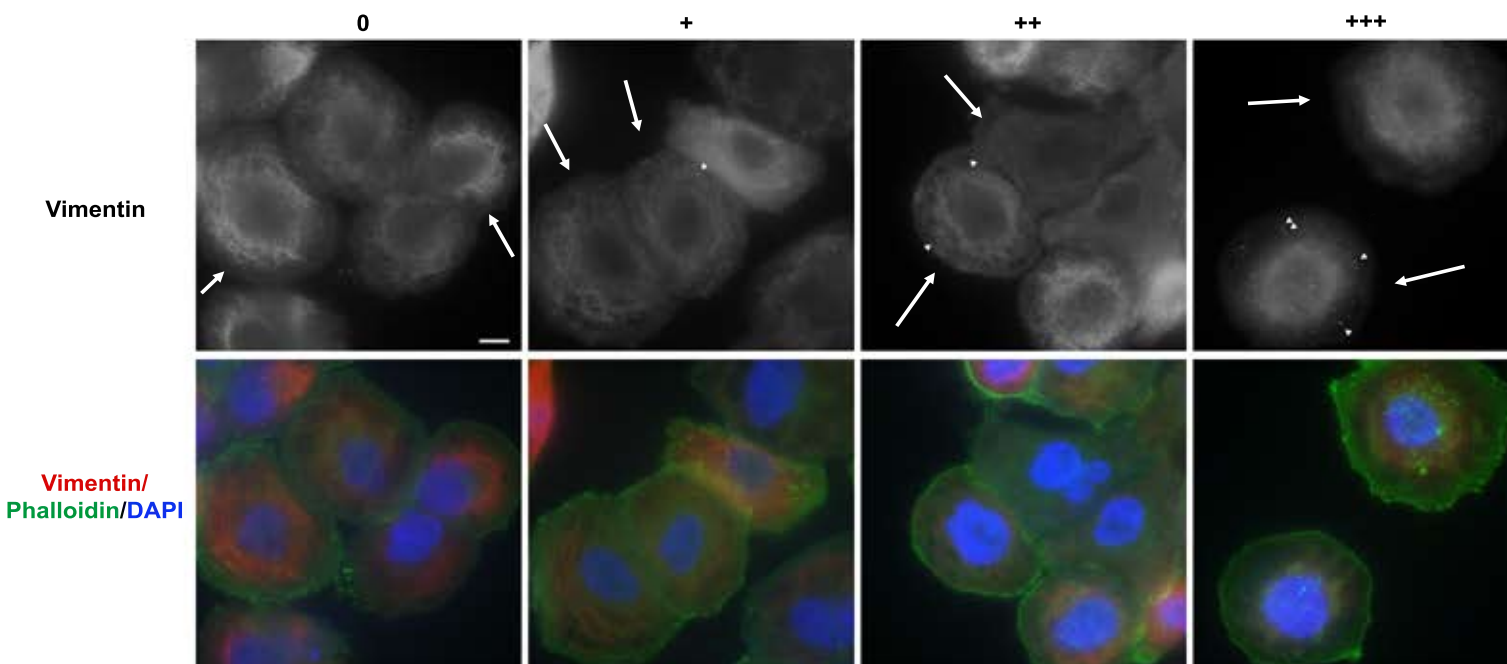


B.

Filament Organization



C.



D.

Filament Fragmentation

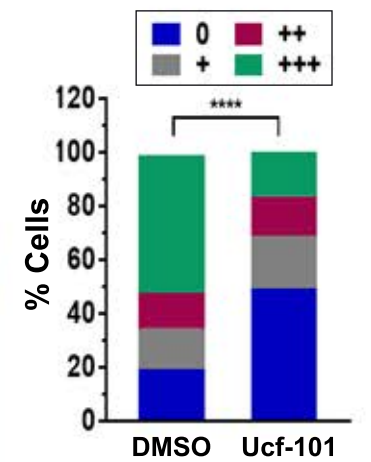
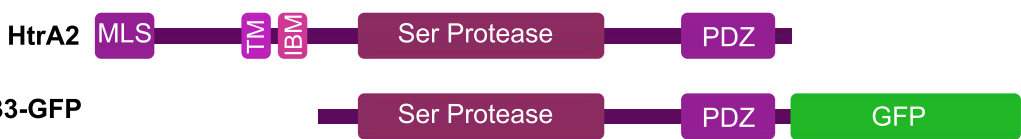
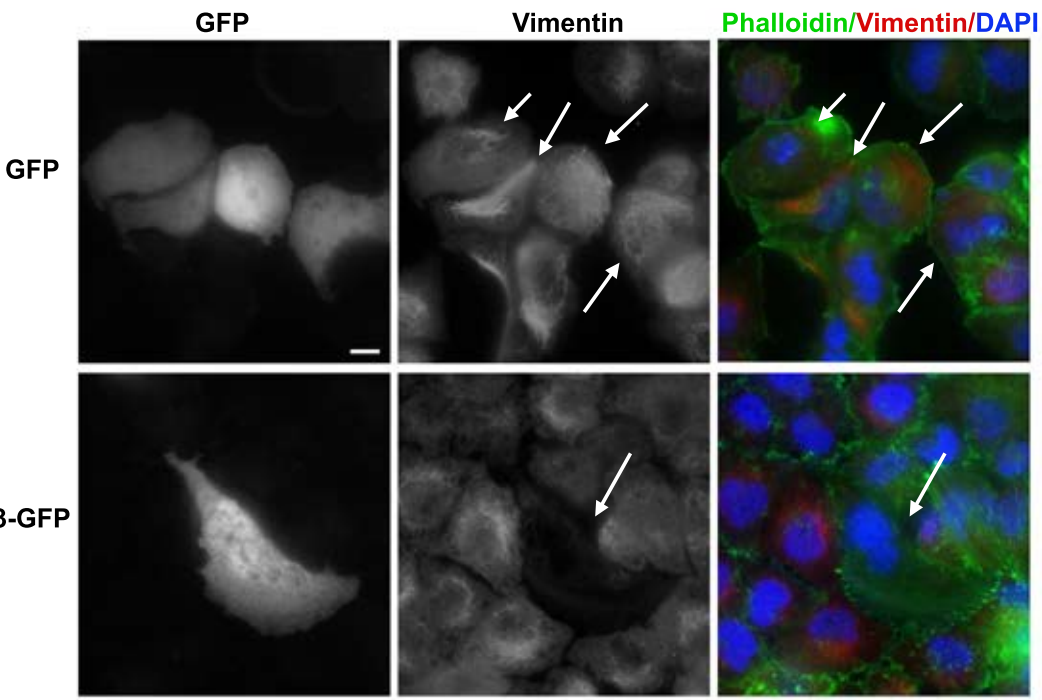


Figure 7

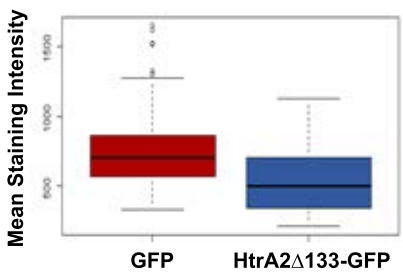
A.



B.



C.



D.

