# Overcoming Pluripotent Stem Cell Dependence on the Repair of Endogenous DNA Damage

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## SUMMARY

Pluripotent stem cells (PSCs) maintain a low mutation frequency compared with somatic cell types at least in part by preferentially utilizing error-free homologous recombination (HR) for DNA repair. Many endogenous metabolites cause DNA interstrand crosslinks, which are repaired by the Fanconi anemia (FA) pathway using HR. To determine the effect of failed repair of endogenous DNA lesions on PSC biology, we generated iPSCs harboring a conditional FA pathway. Upon FA pathway loss, iPSCs maintained pluripotency but underwent profound G2 arrest and apoptosis, whereas parental fibroblasts grew normally. Mechanistic studies revealed that G2-phase FA-deficient iPSCs possess large γH2AX-RAD51 foci indicative of accrued DNA damage, which correlated with activated DNA-damage signaling through CHK1. CHK1 inhibition specifically rescued the growth of FA-deficient iPSCs for prolonged culture periods, surprisingly without stimulating excessive karyotypic abnormalities. These studies reveal that PSCs possess hyperactive CHK1 signaling that restricts their self-renewal in the absence of error-free DNA repair.
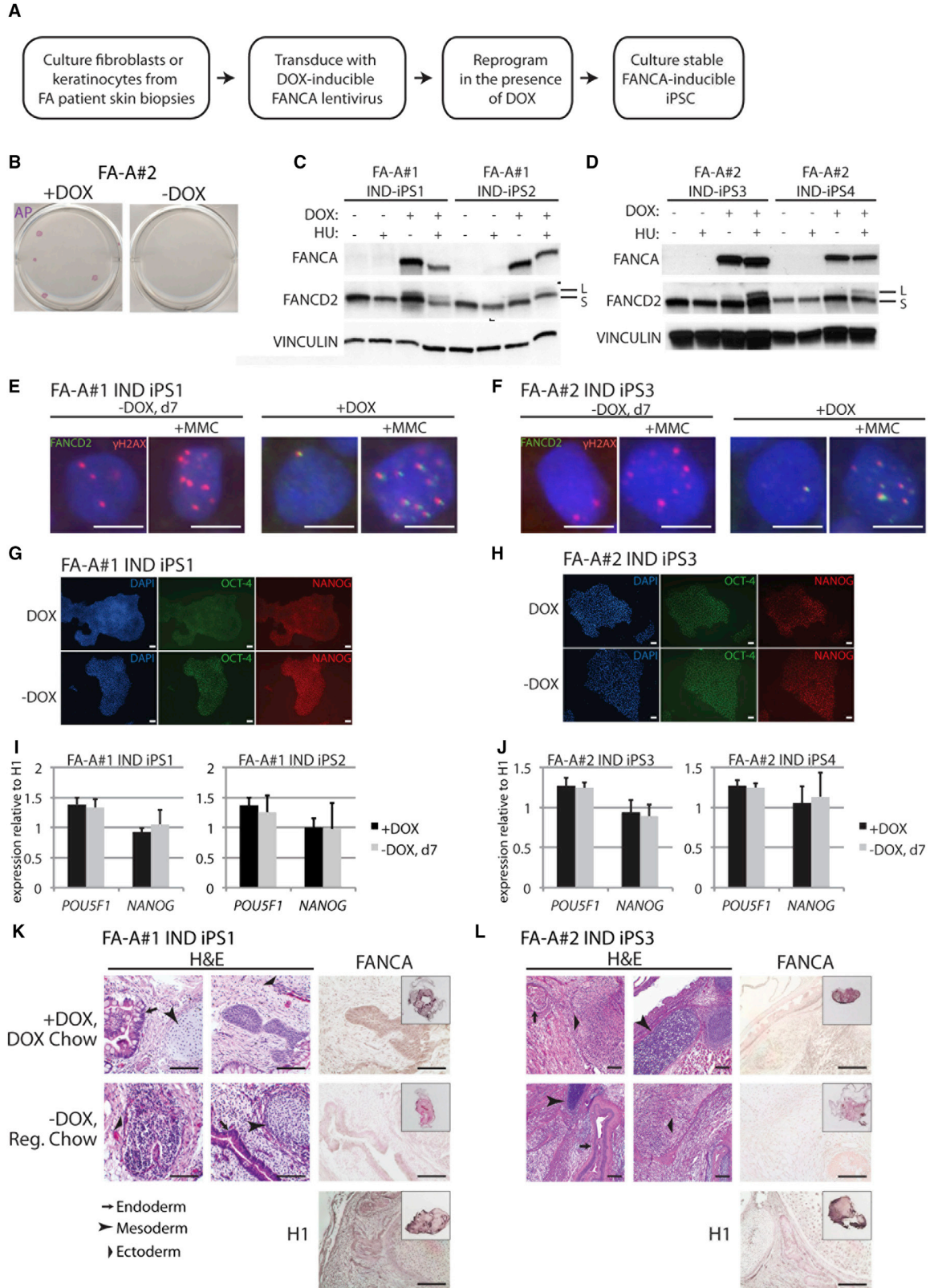
## INTRODUCTION

DNA damage caused by endogenous metabolites is a constant threat to the genomic integrity of all cells. Multiple DNA repair pathways function to identify and repair damage in order to prevent genomic alterations. Embryonic stem cells (ESCs) are unique among all cell types in that mutations in their genomes are potentially propagated throughout the entire organism and germline. It is thought that for this reason they maintain a lower mutation frequency than somatic cells (Cervantes et al., 2002). Previous studies have revealed that ESCs accomplish this by increased scavenging of endogenous sources of DNA damage and increased use of error-free DNA repair pathways, and by undergoing rapid apoptosis when DNA damage levels are elevated (Dannenmann et al., 2015; Momcilovic et al., 2010; Serrano et al., 2011). Many DNA-damaging metabolites, including reactive oxygen species and aldehydes, cause DNA interstrand crosslinks (ICLs). The effect of failed repair of these endogenous lesions on PSC function remains to be explored.

The Fanconi anemia (FA) DNA repair pathway functions at ICLs to recruit DNA repair effector complexes, which prepare the site for error-free repair by homologous recombination (HR) (Kim and D'Andrea, 2012). The pathway consists of a core complex of proteins that serve to identify sites of damage and activate FANCD2, the central effector protein that coordinates downstream repair activities.

Loss-of-function mutations in FA pathway genes cause an inherited disorder characterized by bone marrow failure (BMF) and elevated cancer risk, as well as a myriad of developmental abnormalities (Auerbach, 2009). Diagnosis of FA is determined by sensitivity of patient cells to ICL-inducing agents, such as mitomycin C (MMC), which cause FA cells to undergo cell-cycle arrest or apoptosis and accumulate chromosomal abnormalities (Kim and D'Andrea, 2012). BMF in FA patients is caused by exhaustion of the hematopoietic stem and progenitor cells (HSPC) through cell-cycle arrest and apoptosis (Garaycoechea and Patel, 2013).

Despite the pronounced sensitivity of HSPC to FA pathway dysfunction, other somatic stem cell populations, such as those of the skin and intestine, seem unaffected since their tissues are maintained normally. A number of reports have suggested that pluripotent stem cells (PSCs) are especially dependent on the FA pathway. We have noted previously that both the expression and activity of FANCD2 is dramatically elevated in normally cycling PSCs compared with somatic cells (Chlon et al., 2014). Furthermore, somatic cell reprogramming to induced pluripotent stem cells (iPSCs) was found to be extremely inefficient in FA patient cells due to elevated levels of DNA damage during reprogramming that trigger apoptosis (Chlon et al., 2014; Muller et al., 2012; Raya et al., 2009). Stable inhibition of p53 allowed for the derivation of iPSC colonies from FA patient cells, but these colonies failed to grow into lines, thus suggesting that

CrossMark

**A** Culture fibroblasts or keratinocytes from FA patient skin biopsies → Transduce with DOX-inducible FANCA lentivirus → Reprogram in the presence of DOX → Culture stable FANCA-inducible iPSC

**B** FA-A#2 +DOX / −DOX

**C** FA-A#1 IND-iPS1 / FA-A#1 IND-iPS2
DOX: − − + + − − + +
HU: − + − + − + − +
FANCA
FANCD2 — L / S
VINCULIN

**D** FA-A#2 IND-iPS3 / FA-A#2 IND-iPS4
DOX: − − + + − − + +
HU: − + − + − + − +
FANCA
FANCD2 — L / S
VINCULIN

**E** FA-A#1 IND iPS1
−DOX, d7 / +MMC / +DOX / +MMC
FANCD2 γH2AX

**F** FA-A#2 IND iPS3
−DOX, d7 / +MMC / +DOX / +MMC
FANCD2 γH2AX

**G** FA-A#1 IND iPS1
DOX — DAPI / OCT-4 / NANOG
−DOX — DAPI / OCT-4 / NANOG

**H** FA-A#2 IND iPS3
DOX — DAPI / OCT-4 / NANOG
−DOX — DAPI / OCT-4 / NANOG

**I** FA-A#1 IND iPS1 / FA-A#1 IND iPS2
expression relative to H1
+DOX / −DOX, d7
POU5F1 NANOG

**J** FA-A#2 IND iPS3 / FA-A#2 IND iPS4
expression relative to H1
+DOX / −DOX, d7
POU5F1 NANOG

**K** FA-A#1 IND iPS1
H&E / FANCA
+DOX, DOX Chow
−DOX, Reg. Chow
→ Endoderm
▶ Mesoderm
▸ Ectoderm
H1

**L** FA-A#2 IND iPS3
H&E / FANCA
+DOX, DOX Chow
−DOX, Reg. Chow
H1

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p53-independent signaling limits the self-renewal of FA iPSC (Chlon et al., 2014). Despite this, several recent studies have utilized alternative reprogramming approaches to successfully derive FA patient iPSCs that could be passaged (Liu et al., 2014; Muller et al., 2012; Yung et al., 2013). Others employed short-hairpin RNA (shRNA) or genome engineering to derive FA-deficient ESCs, and although minor growth defects were reported, FA-deficient lines were generated and maintained (Liu et al., 2014; Tulpule et al., 2010). Nonetheless, an important limitation of these studies was that reprogramming or genome engineering require a long clonal growth period during which selection for optimal growth in the absence of FA might occur. Thus, these approaches may underestimate the importance of the FA pathway for PSC growth. Therefore, we sought a conditional approach whereby iPSCs could be derived from FA-proficient cells but then could be studied as isogenic FA-proficient and deficient cell lines after reprogramming.

Here, we describe the derivation of patient-derived iPSCs with inducible complementation of the FA pathway, and report dramatic phenotypes as a consequence of FA pathway loss. Our findings establish that the FA pathway is essential for proliferation and survival of iPSCs and implicates CHK1 as a crucial factor in their extreme sensitivity to accrued DNA damage.

## RESULTS

### Pluripotent Stem Cells Require the FA Pathway for Proliferation and Survival

While there have been several reports of PSC hypersensitivity to exogenous DNA-damaging agents, little is known about the effect of failed repair of endogenous DNA lesions. To investigate this, we generated iPSCs from FA patient cells possessing inducible expression of the complementing FA gene. Somatic cells were isolated from skin biopsies of two FA patients in the FANCA complementation group (Figure 1A). These cells were transduced with a lentivirus expressing the *FANCA* gene under doxycycline (DOX)-inducible control. We confirmed DOX-inducible expression of FANCA, which reconstituted the functional FA core complex to monoubiquitinate FANCD2 upon treatment with hydroxyurea (HU) (Figure S1A). In the absence of DOX, these cells maintained characteristics of FA-deficient cells, including melphalan-induced G2/M arrest and MMC-induced apoptosis (Figures S1B and S1C). We then subjected these cells to reprogramming in the presence or absence of DOX. As expected, only the DOX-treated cells formed iPS colonies (Figure 1B). Several colonies were picked and cultured into stable iPS lines in the presence of DOX. Two independently derived cell lines from each patient were chosen for further study and were named FA-A#1 IND iPS1 and 2, and FA-A#2 IND iPS3 and 4. Once lines were fully established (>10 passages), DOX was withdrawn from the culture to test the inducible system. We confirmed that all lines maintained expression of FANCA in the presence of DOX, whereas FANCA protein was no longer detectable in cells 7 days after DOX withdrawal (−DOX) (Figures 1C and 1D). DOX-treated cells maintained the ability to monoubiquitinate FANCD2 in response to HU treatment (Figures 1C and 1D) and to form FANCD2 foci at γH2AX+ sites of DNA damage in control and MMC-treated cells (Figures 1E and 1F). In contrast, no monoubiquitinated FANCD2 or FANCD2 foci were observed in −DOX cells (Figures 1E and 1F). Despite examining >300 cells per iPSC line, FANCD2 DNA repair foci were never observed in a −DOX cell, confirming that the FA pathway is completely non-functional (Figure S1D). Importantly, monoubiquitinated FANCD2 and FANCD2 foci were detected in +DOX cells in the absence of a DNA-damaging agent, indicating that the FA pathway is active in normal cycling iPSCs (Figures 1C–1F). We assessed the karyotypes of at least 20 metaphases for all lines and found no abnormalities in +DOX cells at passages 10 and 40 post derivation, thus suggesting that genome stability was maintained during iPSC reprogramming and culture (Figure S1H).

Next, we examined whether FA deficiency had an effect on pluripotency. Immunofluorescence (IF) detection of

**Figure 1. Derivation of iPSC with Inducible Complementation of the FA Pathway**
(A) Schematic of derivation of iPSC with inducible complementation.
(B) Alkaline phosphatase (AP) staining of reprogramming cultures in +DOX and −DOX media.
(C and D) Western blot analysis on iPSC formed from FA-A#1 cells and FA-A#2 cells cultured with or without DOX for 7 days. L indicates the larger FANCD2 band, which is the monoubiquitinated form. S indicates the smaller FANCD2 band, which is unmodified.
(E and F) IF staining for FANCD2 and γH2AX foci in iPSC treated with 30 nM MMC for 16 hr. Scale bar, 10 μm.
(G and H) IF for OCT-3/4 and NANOG expression. Scale bar, 300 μm.
(I and J) qRT-PCR for expression of *POU51* and *NANOG* relative to H1 ESCs.
(K and L) H&E staining of teratomas derived from iPSC injected into flanks of NSG mice fed DOX chow or regular chow, respectively. Right-hand panels show immunohistochemistry staining of neighboring sections for FANCA expression. Inset is a 1× magnification of the entire teratoma section. Bottom panels are teratomas derived from H1 ESC in NSG mice fed regular chow. Scale bar, 100 μm.
d7, day 7.

OCT3/4 and NANOG confirmed that both are highly expressed in both +DOX and −DOX cells (Figures 1G, 1H, and S1G). We also assessed the expression of the genes for these proteins, POU5F1 and NANOG, relative to H1 ESCs, and found comparable expression that was unaffected by DOX withdrawal for 7 days (Figures 1I and 1J). Finally, we tested the ability of these cells to form teratomas in +DOX and −DOX conditions. Cells were pre-treated for 4 days with either +DOX or −DOX media and then injected into mice fed DOX chow or regular chow, respectively. All cell lines consistently formed teratomas in both conditions, indicating that pluripotency was unaffected by DOX withdrawal (Figures 1K, 1L, S1E, and S1F). Importantly, we confirmed that the teratomas in DOX chow-fed mice continued to express levels of FANCA similar to teratomas derived from H1 ESCs while teratomas from regular chow-fed (−DOX) mice did not express FANCA. Thus, we conclude that loss of the FA pathway does not affect the pluripotency of iPSCs.

To assess the self-renewal of FA-deficient iPSCs, we monitored their growth over three passages (15 days) and found that the −DOX cultures underwent progressive exhaustion (Figures 2A and 2B). EdU staining to monitor the cell cycle of −DOX cultures revealed a profound cell-cycle arrest in the G2 phase compared with +DOX cultures (Figures 2C and 2D). Statistically significant G2 arrest with a concomitant reduction in S-phase cells occurred in all cell lines (Figures 2E and 2F). We then measured the rates of apoptosis in −DOX compared with +DOX cultures and found a 2- to 4-fold increase in cleaved caspase-3-positive cells, which was further exacerbated by the addition of 30 nM MMC, a dose that caused no detectable apoptosis in fibroblasts (Figures 2G–2J and S1C). Collectively, these data indicate that iPSC depend on the FA pathway for proliferation and survival in standard culture conditions.

## DNA-Damage Signaling Is Activated in FA-Deficient iPSCs and Causes Cell-Cycle Arrest

To determine the cause of the exhaustion of FA-deficient iPSCs, we examined the DNA repair and response activities in +DOX and −DOX cells. First, we detected γH2AX foci, which mark double-strand breaks (DSBs), in cells at each stage of the cell cycle. To do this, we co-stained cells with EdU to label S-phase cells and cyclin B1 (CYCB1) to stain G2 cells. EdU-negative, CYCB1-negative cells were scored as G1. While γH2AX foci were almost never observed in G1-phase iPSCs (<1 per 300 cells), S-phase iPSCs typically harbored numerous, small γH2AX foci. In contrast, a subset of G2 iPSCs possessed a small number (<5) of large γH2AX foci (Figure 3A). The percentage of G2 cells with foci was significantly greater in −DOX than in +DOX cultures, indicating that FA-deficient iPSC accrue DSBs in G2 phase (Figures 3B, 3C, and S2A). Next, we co-stained the

cells for EdU, γH2AX, and RAD51, which is essential for repair of DSB by HR (Figures 3D–3F). We found that nearly all γH2AX foci in EdU-negative −DOX cells stained brightly for RAD51, indicating that the HR machinery was engaged at DSB in the FA-deficient G2 arrested cells. In total, −DOX cells had many more large RAD51 foci than +DOX cells, suggesting that HR-mediated repair is stalled in these cells at a stage beyond RAD51 recruitment (Figures 3E and 3F). Lastly, we co-stained for EdU, γH2AX, and FANCD2 and found that FANCD2 co-localized to many γH2AX foci in EdU-negative +DOX cells but not in −DOX cells, indicating that the FA pathway normally functions at DSB in FA-proficient G2 cells (Figures 3G–3I). Collectively, these data suggest that iPSCs utilize FA and HR-mediated repair during the G2 phase of the cell cycle, and that the inability of FA-deficient iPSCs to repair this damage likely causes G2 arrest.

To examine the effect of this increased DNA damage in FA-deficient iPSCs, we measured the activation of DNA-damage signaling by western blot analysis (Figures 3J, S2B, and S2C). Increases in the activated forms of the DNA-damage sensor kinases ATM and ATR were observed, together with their downstream signal transducers CHK1 and CHK2 in day-7 −DOX cells. These were accompanied by elevated p53 activity and decreased abundance of CDC25A, which is targeted for degradation by CHK1 and required for the G2-M phase transition. Thus, we conclude that DNA-damage signaling is activated in FA-deficient iPSC, likely causing G2 arrest and apoptosis. Importantly, this signaling was not activated in −DOX cultures of the parent FA-A#2 fibroblasts (Figure S2D) despite abundant γH2AX foci in both +DOX and −DOX G2-phase fibroblasts (Figure S2E). Furthermore, the DNA-damage signaling proteins were all expressed at dramatically increased levels in iPSCs when compared with fibroblasts (Figure S2D). Collectively, these data support the notion that iPSCs have a heightened DNA-damage response compared with somatic cells (Dannenmann et al., 2015; Momcilovic et al., 2010), and indicate that DNA damage accrued from endogenous sources activates this signaling.

We next sought to determine whether inhibition of DNA-damage signaling could rescue the growth defects of FA-deficient iPSC. We first attempted rescue using the same shRNA against TP53 as in our previous reprogramming study. Western blot and qPCR indicated that p53 was reduced 80% in sh-TP53 cells and that p53 signaling was significantly repressed (Figures S2F and S2G). However, we found that sh-TP53 only partially rescued the growth of the −DOX cells (Figures S2H and S2I). Cleaved caspase-3-positive cells were reduced in sh-TP53-expressing cells in both +DOX and −DOX cultures, indicating a non-specific decrease in apoptosis (Figures S2J and S2K). In contrast, sh-TP53 had no effect on the G2 arrest of −DOX cells
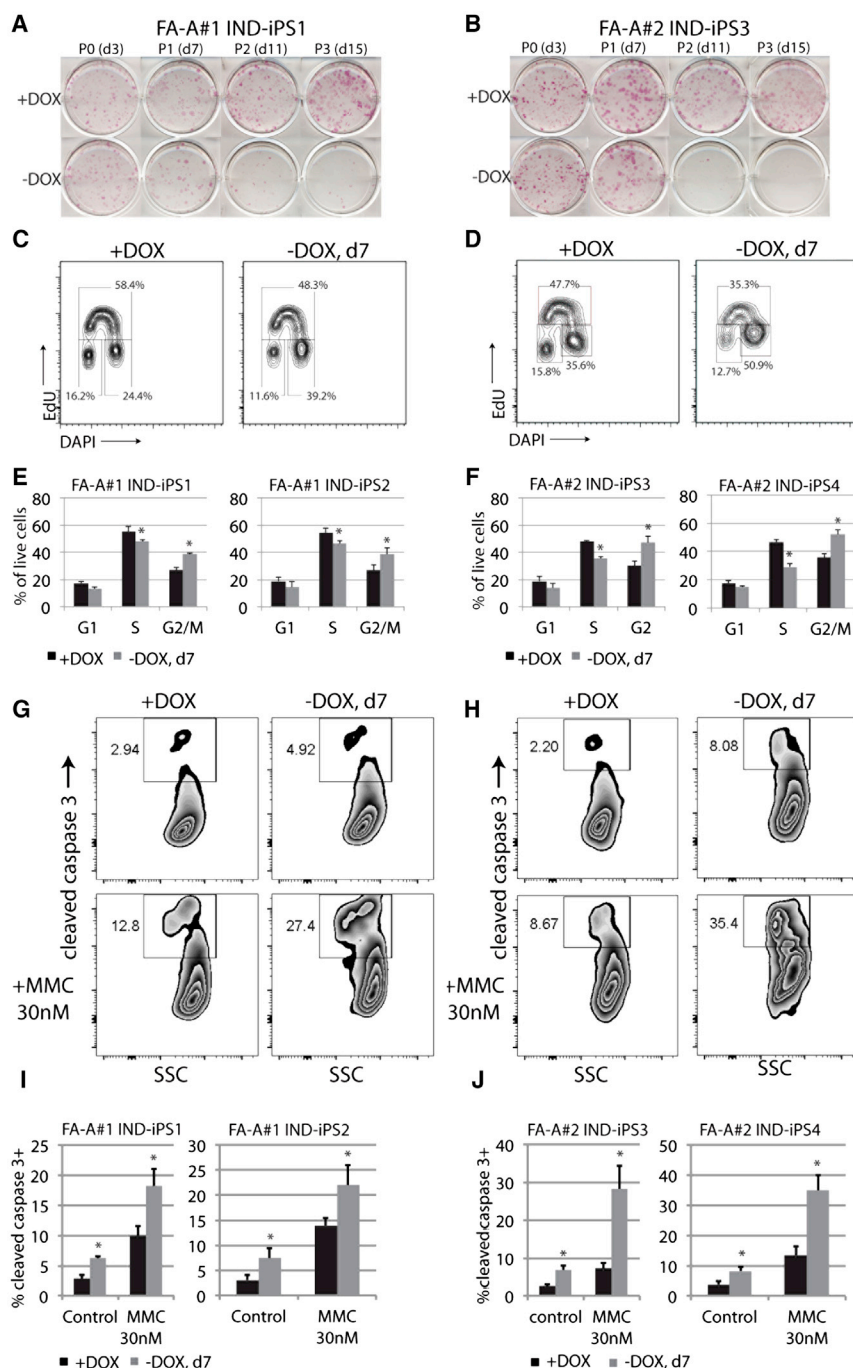
**Figure 2. FA Pathway Loss Causes Progressive Exhaustion of iPSC Cultures**

(A and B) AP staining of iPSC cultured with and without DOX for three passages.

(C and D) EdU cell-cycle analysis of iPSC.

(E and F) Quantification of (C) and (D).

(G and H) Fluorescence-activate cell sorting (FACS) staining for cleaved caspase-3 on iPSC treated or untreated with 30 nM MMC for 24 hr.

(I and J) Quantification of (G) and (H).

d7, day 7. All error bars indicate the standard deviation of the mean. *Statistical significance of $p < 0.05$.

(Figures S2L and S2M). These data indicate that p53 does not affect G2 arrest in FA-deficient cells.

We next sought to identify mechanisms of G2 arrest induction in FA-deficient iPSCs by screening a series of inhibitors targeting DNA-damage response proteins. Inhibitors were first tested for the ability to allow arrested G2 cells in −DOX cultures to escape, undergo mitosis, and cycle into G1. To do this, we used a short inhibitor treat-

ment of 2.5 hr, stained for EdU in the last 45 min, and examined the cell-cycle profile. The ATM inhibitor KU55933 had no effect on the G2 arrest (Figures 3K and S2N). The ATR inhibitor ETP-46464 allowed some cells to escape G2 in a dose-dependent manner (Figures 3K and S2N), but also caused significant cell death in both +DOX and −DOX cultures (data not shown). Remarkably, the CHK1 inhibitor AZD7762 (CHK1i)
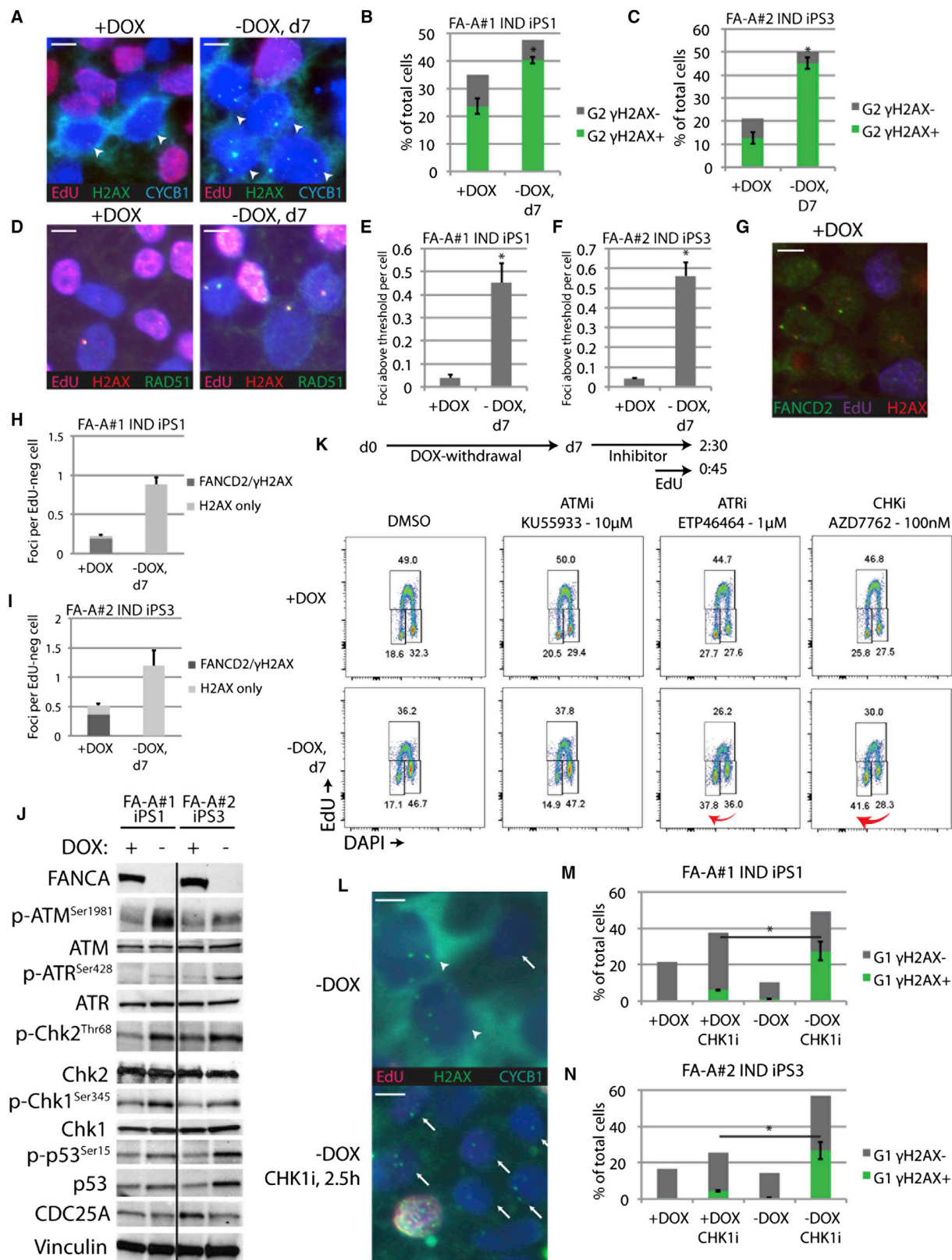
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allowed full recovery of the −DOX G2 cells back to the same level as +DOX cultures and resulted in an accumulation of G1 cells, which was expected since escaping G2 cells did not have enough time to enter S phase (Figure 3K). The effect of CHK1i was dose dependent, and 100 nM allowed full recovery of the G2 arrest (Figure S2N). These data indicate that CHK1 inhibition rescues the G2 arrest of FA-deficient iPSC.

Next, we examined the cells for DSBs after a 2.5 hr treatment with 100 nM CHK1i. As expected, we found an abundance of G1 cells in the CHK1i-treated −DOX culture, but surprisingly these G1 cells possessed γH2AX foci (Figure 3L), whereas G1 cells in untreated cultures never had γH2AX foci and few CHK1i-treated +DOX cells had foci (Figures 3M and 3N). This result suggests that the CHK1i allows the G2-arrested cells to undergo mitosis without repairing existing DNA damage.

### Inhibition of CHK1 Completely Restores Growth of FA-Deficient iPSC

Based upon the sustained presence of DNA repair foci in CHK1i-treated FA-deficient cultures, the expectation was that cellular growth would be limited by the rapid acquisition of chromosomal damage and consequent cell death. We thus cultured the FA-inducible iPSCs in CHK1i for a prolonged period of time. After three passages (15 days) in −DOX conditions, CHK1i fully restored the growth of the −DOX cultures to the same level as +DOX cultures (Figures 4A and 4B). There was no effect on the growth of +DOX cells, indicating a specific effect on the growth of FA-deficient iPSC. CHK1i treatment led to a dramatic increase in the phosphorylation of CHK1-Ser345, and increased phosphorylation of ATR-Ser428 (Figure 4C). In contrast, CHK2 activation was unaffected by the CHK1i. Downstream of CHK1, we found that CDC25A levels were greatly increased and Ser-15 activation of p53 was fully attenuated by CHK1i-treatment. These findings are consistent with decreased G2 arrest and p53-mediated

apoptosis, respectively. Cell-cycle and apoptosis analysis of +DOX and −DOX cultures treated with CHK1i for 72 hr revealed a complete recovery of G2 arrest and apoptosis in both cell lines (Figures 4D–4I and S3A–S3D). To confirm that inhibition of CHK1 is responsible for the rescue, we used MK-8776, a selective CHK1 inhibitor, and found that it also fully rescued the G2 arrest (Figures S3E and S3F).

To determine the level of genome instability caused by prolonged CHK1 inhibition, we assessed the karyotypes of iPSCs grown in the presence and absence of both DOX and CHK1i for more than 40 days. The −DOX untreated cells were rarely passaged in order to maintain enough cells for analysis. The CHK1i-treated +DOX and −DOX cells were passaged every 4 days and grew similarly to +DOX untreated cells (Figure 4J). From each condition, 20–40 metaphases were scored for both lines, and both untreated and CHK1i-treated +DOX cells had normal karyotypes (Figure 4K). For the untreated −DOX cells, we observed 2 cells out of 20 with trisomy 12 in the FA-A#1 iPS1 cells and no abnormalities in the FA-A#2 iPS3 cells. For the CHK1i-treated −DOX cells, we observed trisomies in only 2 cells out of 40 for the FA-A#1 iPSCs, and translocations in only 1 cell out of 40 on chr4 and chr5 for the FA-A#2 iPSCs. The absence of profound karyotypic abnormalities coupled with the stable growth of CHK1-inhibted iPSCs suggests that other error-free repair pathways compensate for FA deficiency when CHK1 is inhibited.

## DISCUSSION

In this report, we demonstrate that human iPSCs require the FA pathway for efficient growth in vitro, and establish CHK1 as a critical regulator of iPSC hypersensitivity to DNA damage. The FA pathway is active in S- and G2-phase iPSCs, where it likely functions to repair DNA damage caused by

**Figure 3. Unrepaired DNA Damage Causes Cell-Cycle Arrest in FA-Deficient iPSCs through CHK1 Signaling**
(A) IF on EdU-stained iPSC for γH2AX and CYCB1. CYCB1 marks G2 cells (arrowheads).
(B and C) Quantification of the percentage of total cells in G2 with and without γH2AX foci.
(D) IF on EdU-stained iPSC for γH2AX and RAD51.
(E and F) Quantification of RAD51 foci per cell above an empirical threshold.
(G) IF on EdU-stained cells for γH2AX and FANCD2.
(H and I) Quantification of FANCD2/γH2AX double-positive foci and γH2AX only positive foci in EdU-negative cells.
(J) Western blot on whole-cell lysates from iPSC cultured with and without DOX for 7 days.
(K) iPSC cultured without DOX for 7 days were treated with inhibitors for 2.5 hr and pulsed with EdU for the last 45 min. Red arrows indicate inhibitors that allow G2-arrested cells in −DOX cultures to move to G1.
(L) IF for EdU, γH2AX, and CYCB1 on day-7 −DOX iPSC treated with 100 nM CHK1i for 2.5 hr. Arrowheads indicate G2 cells and arrows G1 cells.
(M and N) Quantification of the percentage of total cells in G1 with and without γH2AX foci.
d7, day 7. All error bars indicate the standard deviation of the mean. *Statistical significance of $p < 0.05$. Scale bars, 10 μm.
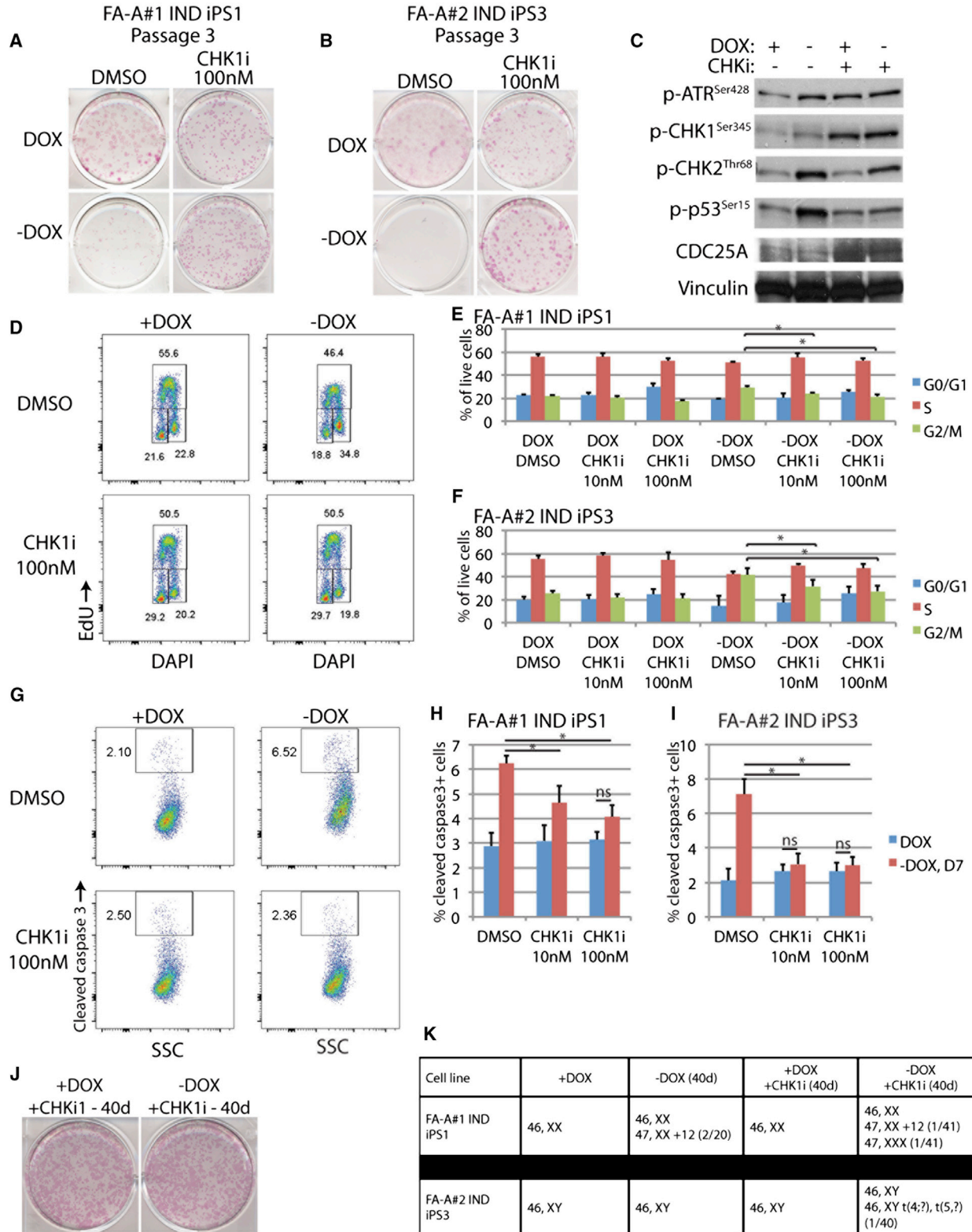
**Figure 4. Inhibition of CHK1 Completely Rescues Growth Defects of FA-Deficient iPSC**

(A and B) AP staining of iPSC cultured with CHK1i or DMSO for three passages.

(C) Western blot on iPSC treated with 100 nM CHK1i for 72 hr.

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endogenous sources. Unrepaired damage coincides with profound G2 arrest and apoptosis in FA-deficient iPSCs, whereas somatic cell types with greater levels of damage cycle normally. Surprisingly, inhibition of CHK1 completely and specifically rescues growth of FA-deficient iPSCs without causing extensive karyotypic abnormalities. This suggests that CHK1 is abnormally heightened in iPSCs and that dampening CHK1 activity may be possible without excessive damage accrual. The risks associated with CHK1 inhibition must now be assessed in experimental models using methods more sensitive than karyotyping.

Analysis of DNA-damage foci in CHK1i-treated cells led to the remarkable discovery that CHK1i allowed FA-deficient iPSCs to escape G2 arrest and undergo mitosis without repairing the DSBs that had evidently caused their arrest. A previous study indicates that DSBs arising during G2-M phase are bound together by the MRN complex to be repaired after mitosis (Giunta et al., 2010). The damage is then repaired in G1 by error-prone non-homologous end-joining (NHEJ) or in the subsequent S phase by HR. In the CHK1-inhibited iPSCs, HR is unlikely since CHK1 phosphorylation of RAD51 is required for HR (Sorensen et al., 2005). NHEJ is thus likely to compensate for the FA pathway, but it is predicted to cause chromosomal translocations, which we rarely observed in the FA-deficient iPSCs. Future studies are necessary to determine whether these or other repair pathways compensate for FA in CHK1-inhibited iPSCs.

A recent report indicates that BRCA1 is required for ICL-induced activation of CHK1 in FA-deficient cells, indicating that the engagement of the HR machinery upstream of the FA pathway plays a role in checkpoint signaling (Draga et al., 2015). Correspondingly, we observed that HR proteins were engaged at damage foci in G2-phase FA-deficient iPSCs by the localization of RAD51. Since iPSCs are so profoundly dedicated to HR-mediated repair, increased BRCA1 activity may contribute to the hypersensitivity of CHK1 in this cell type.

FA is a complex disease that presents with a myriad of congenital abnormalities, indicating that FA affects the development and maintenance of diverse tissues in utero, including but not limited to the hematopoietic system. Fetal or post-natal therapeutic intervention for these developmental defects is not currently available. The conditional FA iPSC model can now provide an experimental platform for discovering new drug targets to sustain stem cell fitness in multiple tissues in the context of FA. CHK1 inhibition is of interest in this regard, given its potential for stimulating the growth of FA hematopoietic cells (Ceccaldi et al., 2011) and current clinical use for the targeting of epithelial tumors, which are common in FA patients. While CHK1 repression bears a risk of introducing mutations through the abrogation of checkpoint control, a therapeutic window should now be explored for the use of CHK1 inhibitors to prevent pre- and post-natal FA phenotypes.

## EXPERIMENTAL PROCEDURES

### Cell Culture

Patient skin biopsies were obtained from the FA Tissue Repository at Cincinnati Children's Hospital (IRB #2008-1331). Patient keratinocytes and fibroblasts were cultured from fresh skin punch biopsies as described by Chlon et al. (2014). Biopsy FA-A#1 was cultured into keratinocytes, and biopsy FA-A#2 was cultured into fibroblasts. pInducer20-FANCA lentiviral construct was derived by Gateway cloning of the full-length human *FANCA* cDNA from a pDONR plasmid (Harvard plasmID) into pInducer20 (Meerbrey et al., 2011). Keratinocytes or fibroblasts were transduced with pInducer20-FANCA and then selected with G418 for 3 days. For reprogramming, keratinocytes were transduced with a polycistronic lentivirus expressing OCT-4, SOX2, KLF4, C-MYC, and RFP (Warlich et al., 2011). Fibroblasts were reprogrammed by episomal transfection using the Amaxa system (Lonza) with four plasmids expressing GFP, OCT-4 and sh-*TP53*, SOX2 and KLF4, and MYC and LIN28 (Addgene 27082, 27077, 27078, 27080), as described previously (Okita et al., 2011). At 4 days post-transfection with reprogramming factors, the cells were plated on irradiated CF-1 MEFs (GlobalStem) in hESC medium containing 100 ng/ml DOX. After 20 days, colonies with characteristic iPSC morphology were picked and transferred to dishes coated with Matrigel (BD Biosciences) in mTeSR1 (STEMCELL Technologies) with 100 ng/ml DOX. mTeSR1 medium was changed daily and colonies were passaged as needed (about every 4 days) using Dispase, as described previously (Chlon et al., 2014). All analyses were done on cells cultured without DOX for 7 days.

(D) EdU cell-cycle analysis of iPSC with and without CHK1i for 72 hr.
(E and F) Quantification of (D).
(G) FACS staining for cleaved caspase-3 on iPSC cultured with CHK1i or DMSO for 72 hr.
(H and I) Quantification of (G). D7, day 7.
(J) AP staining of iPSC cultured with 100 nM CHK1i for 40 days.
(K) Chart indicating karyotypic abnormalities observed in iPSC cultured with and without DOX, and with and without 100 nM CHK1i, for 40 days. Proportion of metaphases counted with a specific defect are listed in parentheses. Where not indicated, 20 metaphases were counted.
ns, not significant. All error bars indicate the standard deviation of the mean. *Statistical significance of $p < 0.05$.

## Immunofluorescence

For IF, cells were fixed, permeabilized, and stained as described previously (Chlon et al., 2014). Where indicated, 30 nM MMC was added to the medium 16 hr before fixation. For EdU staining, cells were incubated with 10 μM EdU for 45 min before fixation. EdU Click-IT Imaging kits were used following the manufacturer's instructions (Life Technologies) followed by standard IF protocol. For quantification of IF, >100 cells were counted from three independent biological replicates.

## Flow Cytometry

For cell-cycle analysis, cells were incubated with 10 μM EdU for 45 min before fixation and the Click-it EdU Cell Proliferation Assay kit (Life Technologies) was used for staining. For cleaved caspase-3 staining, PFA-fixed cells were permeabilized and stained with an Alexa 647-conjugated antibody (Cell Signaling) following the manufacturer's protocol.

## Teratoma Formation

Teratoma formation was carried out as described previously in NSG mice (Chlon et al., 2014). Cells were maintained in either +DOX or −DOX conditions for 4 days before harvesting and then were injected into mice fed chow containing DOX or regular chow, respectively.

## Statistics

All pairwise comparisons were examined for statistical significance by Student's t test with $p < 0.05$. Error bars indicate the SD of the mean for three independent biological replicates.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2015.12.001.

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