HHS Public Access

Author manuscript

Cell Stem Cell. Author manuscript; available in PMC 2016 June 04.

Published in final edited form as:

Cell Stem Cell. 2015 June 4; 16(6): 684–698. doi:10.1016/j.stem.2015.03.017.

Filia is an ESC-specific regulator of DNA damage response and safeguards genomic stability

Bo Zhao[1,2], **Wei-dao Zhang**[1,2,3], **Ying-liang Duan**[1,2], **Yong-qing Lu**[1,2], **Yi-xian Cun**[1,2], **Chao-hui Li**[1,2,3], **Kun Guo**[1,2,3], **Wen-hui Nie**[1], **Lei Li**[4], **Rugang Zhang**[5], and **Ping Zheng**[1,2,*]

[1] State Key Laboratory of Genetic Resources and Evolution, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China

[2] Yunnan Key Laboratory of Animal Reproduction, Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, Yunnan 650223, China

[3] Kunming College of Life Science, University of Chinese Academy of Sciences, Kunming 650204, China

[4] State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing, 100101, China

[5] Gene Expression and Regulation Program, The Wistar Institute Cancer Center, The Wistar Institute, Philadelphia, PA 19104, United States

Summary

Pluripotent stem cells (PSCs) hold great promise in cell-based therapy, but the genomic instability seen in culture hampers full application. Greater understanding of the factors that regulate genomic stability in PSCs could help address this issue. Here we describe the identification of Filia as a specific regulator of genomic stability in mouse embryonic stem cells (ESCs). Filia expression is induced by genotoxic stress. Filia promotes centrosome integrity and regulates DNA damage response (DDR) through multiple pathways, including DDR signaling, cell cycle checkpoints and damage repair, ESC differentiation and apoptosis. Filia depletion causes ESC genomic instability, induces resistance to apoptosis and promotes malignant transformation. As part of its role in the DDR, Filia interacts with PARP1 and stimulates its enzymatic activity. Filia also constitutively resides on centrosomes and translocates to DNA damage sites and mitochondria, consistent with its multifaceted roles in regulating centrosome integrity, damage repair and apoptosis.

Introduction

Pluripotent stem cells (PSCs) hold great potential for cell-based regenerative medicine. However, genomic instability and tumorigenicity limit their full applications. Understanding the mechanisms that regulate their genome stability is critical to address this issue. These mechanistic insights are also important to understand how pluripotent cells (e.g. germ cells and early embryos) sustain their genome integrity to ensure the successful development of

[*]**Contact to**: Ping Zheng, zhengp@mail.kiz.ac.cn.

an organism. Pluripotent cells are capable of developing into all cell types, whereas somatic cells are cell-fate restricted. Accordingly, pluripotent cells possess higher competence than somatic cells to protect their genetic integrity.

DNA damage response (DDR) is a fundamental and evolutionarily conserved mechanism to preserve genomic integrity of cells (Behrens et al., 2014; Jackson and Bartek, 2009). Upon DNA damage triggered by endogenous or exogenous insults, cells elicit complicated and highly coordinated response networks, including damage sensing and signal transduction, which trigger cell cycle arrest and DNA repair. When the extent of DNA damage is beyond repairable, cells undergo apoptosis or senescence to prevent the passage of the mutations to descendent cell populations. These responses are coordinated at multiple levels of gene regulation including at the transcriptional, post-transcriptional, translational and posttranslational levels. Recent advances have further extended our understanding of the DDR by documenting cytoplasmic Golgi dispersal as a novel component of the DDR network (Farber-Katz et al., 2014). Due to the importance of DDR in genomic stability, its dysfunction is closely associated with genetic diseases, tumorigenicity, and tissue aging (Bartkova et al., 2005; Liang et al., 2009; Rass et al., 2007). DDR has been intensively studied in somatic cells and many key players have been identified. Compared to somatic cells, very few studies have been conducted in pluripotent cells regarding their DDR network components. Limited reports suggested that PSCs employed distinct strategies to cope with DNA damage (Wyles et al., 2014). For instance, mouse ESCs bypass the G1/S cell cycle checkpoint due to a extremely short G1 phase (van der Laan et al., 2013). Instead, intra-S and G2 cell cycle checkpoints are critical for ESCs (Momcilovic et al., 2011). PSCs predominantly employ error-free homologue recombination (HR) rather than error-prone non-homologous end joining (NHEJ) pathway to repair DNA double strand break (DSB) (Tichy et al., 2010). Moreover, PSCs utilize high mitochondrial priming and retention of constitutively active Bax at the Golgi to sensitize them to DNA damage (Dumitru et al., 2012; Liu et al., 2013). Although it is appreciated that DDR regulation in PSCs is distinct from that in somatic cells, the key players and their functional mechanisms remain unknown. In particular, PSC-specific DDR factors have never been identified.

Filia (official name, KH domain containing 3; also known as *Ecat1*) was first identified in mESCs (Mitsui et al., 2003). Its expression is restricted to undifferentiated ESCs. Later studies reported its expression in growing oocytes and identified two transcriptional isoforms. The long isoform (~1.6 kb) encodes a ~70KD protein and is predominantly expressed in ESCs, while the short isoform (~1.2 kb), encoding a ~50KD protein, is primarily detected in growing oocytes (Ohsugi et al., 2008). Functional analysis revealed that *Filia* is not essential for ESC self-renewal (Mitsui et al., 2003), whereas depletion of maternal Filia protein in oocytes led to severe aneuploidy in cleavage stage embryos (Zheng and Dean, 2009). Here we report Filia acts as a mESC-specific regulator of DDR and safeguards genomic stability.

Results

Loss of Filia causes genomic instability and promotes malignant transformation of mESCs

To investigate the role of Filia in regulating genomic stability of mESCs, we derived three *Filia*$^{-/-}$ ESC lines (FK(I), FK(II), and FK(III)) and two wild-type (WT) counterparts from the *Filia* targeted mutant mice (Zheng and Dean, 2009). The success rates of ESC derivation did not differ between mutant and WT blastocysts (33.3% [2/6] in WT versus 25% [3/12] in *Filia* mutant), indicating that Filia is not required for the derivation of ESCs. Consistent with previous studies (Mitsui et al., 2003), loss of *Filia* did not impair the self-renewal of ESCs. FK ESCs displayed comparable morphology, expression of pluripotency markers, alkaline phosphatase staining, formation of embryonic bodies, cell cycle profiles, and growth competition ability compared with WT cells (Figures S1A-S1C). No overt morphological abnormality was observed in FK ESCs after 100 passages.

However, cytogenetic analysis of chromosome metaphase spreads revealed that FK ESCs exhibited severe chromosome abnormalities, including chromosome breaks (Figures 1A and 1B), fusion of chromosome ends (Figures 1A, 1C and 1D), and sister chromatid exchange (SCE) (Figure 1E). Consistently, FK ESCs displayed a higher rate of chromosomal anaphase bridges (Figures 1B and 1F) and an increase in DNA damage markers such as γH2AX accumulation and foci formation (Figures 1G and 1H). The increase in DNA damage in FK ESCs was further validated by comet assay, a method that measures the extent of DNA damage on a single cell basis (Figure 1I). Moreover, FK ESCs had high incidences of abnormal centrosomes, spindle assembly (Figure 1J) and aneuploidy (Figures S1D and S1E). These phenotypes were reproducibly observed in another ESC line with distinct genetic background in which *Filia* expression was knocked down by an inducible shRNA (Figures S2A-S2E), indicating they are not genetic background specific.

Genomic instability is known to promote cell transformation and tumorigenesis. Accordingly, we assessed the tumorigenicity of FK ESCs by injecting the unlabeled FK and GFP-labeled WT ESCs into the right or left testis of the same NOD/SCID mouse. Teratomas formed by FK ESCs (GFP^{-}) weighed more than those formed by WT ESCs (GFP^{+}) (Figures 1K-1M). Furthermore, aggressive tumors lacking GFP expression were detected in pancreas (Figure 1N), suggesting they were formed by FK ESCs. Consistently, FK ESCs showed a delay in differentiation (Figure S1F). Thus, we conclude that loss of Filia causes genomic instability and promotes tumorigenesis.

Filia is induced by genotoxic insults and is required for activation of ATM and Chk2

Since FK ESCs displayed a severe DNA damage phenotype under normal culture condition, we sought to determine whether Filia regulates DDR. Toward this goal, we investigated if Filia expression is regulated by genotoxic insults. Indeed, the 70 KD Filia was up-regulated by DNA damaging agents such as ultraviolet light (UV), etoposide, doxorubicin, camptothecin, and hydroxyl urea (Nagy and Soutoglou, 2009) (Figure 2A). The 50 KD isoform was not detected in ESCs (Figure S3A). The expression and up-regulation of Filia was specific to ESCs, as mouse embryonic fibroblasts (MEFs) and mesenchymal stem cells (MSCs) did not express Filia in either untreated or treated condition (Figure S3B).

We then systematically investigated the effects of *Filia* loss on major DDR signaling components, including γH2AX, ATM, ATR, Chk1, Chk2, and p53. Upon etoposide treatment, γH2AX, p-ATM, p-ATR, p-Chk1, and p-Chk2 were induced and sustained for at least 12 hr in WT ESCs (Figure 2B). In contrast, γH2AX and p-ATM was initially induced at comparable levels in FK and WT ESCs, but failed to sustain in FK cells (Figures 2B and S3C). Strikingly, Chk2, one of the key substrates of ATM, was not phosphorylated at all in FK ESCs (Figure 2B). This suggests that Filia participates in the Chk2 activation via mechanisms independent of ATM. Unlike the ATM-Chk2 signaling axis, ATR and Chk1 kinases were not significantly affected by Filia loss (Figures 2B and S3D). ATM/Chk2 regulates p53 activity. Consistently, phosphorylation of p53 at its S15 and S20 was impaired in FK ESCs compared to WT ESCs (Figure 2B). To further define the domain necessary for regulating the signal transduction, we stably expressed full length Filia, Filia fragment containing amino acids (aa) 1-340, or KH domain (atypical RNA-binding domain) containing aa1-125, in FK ESCs (FK-Filia, FK-340, and FK-KH rescue cells, respectively) (Figures S3A, S3E, and S3F). The induction of γH2AX, p-ATM, p-Chk2, and p-p53 were restored in FK-Filia and FK-340 (Figure 2B), but not in FK-KH cells (Figure S3G). These data revealed that C-terminus of Filia (aa341-440) was dispensable for DDR signaling. Moreover, these functions were independent of genetic background based on shRNA knock-down ESCs as described above (Figure S2A).

In somatic cells, ATM and Chk2 activation exhibited pulsatile dynamics in response to DNA damage. Recurrent initiation of ATM/Chk2 activation is an important mechanism to sustain DDR (Batchelor et al., 2008). To better understand the dynamics of ATM/Chk2 activation in ESCs and the influence of *Filia* loss on ATM and Chk2 activation, we conducted a detailed time-course analysis. WT ESCs displayed two waves of ATM activation. Filia loss did not affect the initial ATM activation between 1-4 hr post damage, but completely abolished ATM activity thereafter (Figure 2C). This suggests that distinct mechanisms regulate two phases of ATM activation, with the second phase relies on Filia. Unlike ATM, Chk2 activation did not exhibit discrete phases in ESCs. Moreover, Filia loss completely blocked Chk2 activation (Figure 2C), implying that Filia is necessary for Chk2 activation. Of interest, Filia expression exhibited a similar oscillation pattern as ATM activation in WT ESCs (Figure 2C). To further explore the relationships among Filia, ATM, and Chk2, we inhibited the ATM kinase activity using a specific ATM inhibitor KU55933 and examined Filia expression and Chk2 activation. Inhibition of ATM activity did not affect Filia expression but impaired Chk2 activation (Figure S3H). These data suggests that Filia functions upstream of ATM in DDR.

Filia regulates cell cycle checkpoints and DNA damage repair

Cell cycle checkpoint and DNA repair rely on DDR signaling (Branzei and Foiani, 2008). For instance, Chk1 is required for initiation of G2/M checkpoint in mESCs (Liu et al., 2000), and Chk2 is required for maintenance of G2/M arrest (Hirao et al., 2000). ATM is critical for both G2/M checkpoint and DNA repair in ESCs (Momcilovic et al., 2009; Yamamoto et al., 2012). Cell cycle analysis revealed that S and G2/M checkpoints were impaired in FK ESCs, which could be rescued by Filia (Figures 3A and 3B). Intriguingly, expression of Filia340 failed to restore cell cycle checkpoints despite its ability to rescue

DDR signaling (Figures 3A and 3B). Thus, cell cycle checkpoint defects in FK ESCs were not simply a consequence of DDR signaling failure. Rather, Filia itself participated in the regulation of cell cycle checkpoints, and this function required its C-terminus (aa 341-440).

ATM activation is essential for DNA damage repair (Yamamoto et al., 2012). To investigate if Filia loss impairs DNA damage repair, we performed comet assay to evaluate the repair competence in WT, FK, FK-Filia and FK-340 rescue ESCs. ESCs were treated with etoposide and the extent of DNA damage was evaluated after 0 hr, 6 hr and 12 hr of recovery. Notably, WT, FK-Filia and FK-340 cells displayed similar competence in DNA damage repair, whereas FK cells exhibited an impairment in damage repair (Figure 3C). Although Filia340 was less efficient than Filia at 6 hr, this difference disappeared at 12 hr post recovery (Figure 3C). Consistently, FK-Filia and FK-340 ESCs showed similarly mild DNA damage when compared to WT cells (Figure 3D), supporting the notion that re-introduction of Filia or Filia340 into FK ESCs is sufficient to restore the DNA damage repair. Rad51 is a key protein involved in HR-mediated DSB repair. In WT as well as FK-Filia and FK-340 rescue ESCs treated with etoposide, most γH2AX foci were co-localized with the Rad51 foci when examined at 12 hr post damage (Figure 3E). However, in FK ESCs, the number and size of Rad51 foci decreased and there were a significant number of γH2AX foci lacking co-localized Rad51 (Figure 3E). Notably, the recruitment of active DNA-PKcs to DNA damage sites, a marker of NHEJ-mediated DSB repair (Davis et al., 2014), was not affected Filia loss (Figure S3I). This suggests that Filia depletion preferentially impairs HR-mediated DSB repair. Rad51 protein was expressed at comparable levels among WT, FK, FK-340 or FK-Filia ESCs (Figure S3J), implying that Filia facilitates the efficient recruitment of Rad51 to the damage sites. It is intriguing that Filia340 rescued cells are capable of repairing DNA damage, despite the lack of cell cycle checkpoints. This suggests that cell cycle arrest is not a prerequisite for DNA damage repair and the two processes are regulated independently.

Filia regulates differentiation and apoptosis of ESCs in response to DNA damage

Elimination of cells with irreparable DNA damage is the last and most critical safeguarding event in DDR. Stem cells display hypersensitivity to DNA damage (Dumitru et al., 2012; Liu et al., 2013) and are primed to undergo rapid differentiation and apoptosis to ensure the genome stability (Inomata et al., 2009; Lin et al., 2005). FK ESCs did not encounter culture crisis despite accumulated spontaneous DNA damage, suggesting the hypersensitivity to DNA damage is impaired in these cells. To test this hypothesis, we treated the ESCs with etoposide and investigated the dynamics of differentiation and apoptosis. Sub-G1 apoptotic cell analysis revealed that FK ESCs were more resistant to cell death than WT ESCs (Figure 4A). Consistently, there was a decrease of cleaved caspase-3, a critical executioner and marker of apoptosis, in FK compared to WT ESCs (Figure 4B). This phenotype was rescued by Filia, but not Filia340 (Figures 4A and 4B). This result indicates that the C-terminus of Filia (aa 341-440) was necessary for this function. ESC differentiation is triggered by transactivation of p53 that binds to the enhancer region of *Nanog* to suppress its expression (Li et al., 2012; Lin et al., 2005). Consistently, Nanog, but not Oct4, displayed significant down-regulation at 12 hr after damage in WT and FK-Filia ESCs, but not in FK or FK-340 cells (Figure 4C).

To more comprehensively evaluate the functional outcome of *Filia* loss on cell survival in response to DNA damage, we performed clonal competition assay in which same numbers of two type ESCs were mixed, exposed to DNA damage and co-cultured for six days. To distinguish the two mixed cell types, one was labeled with GFP expression. Compared with WT ESCs, FK cells showed a higher survival rate (Figure 4D). Furthermore, WT, but not FK ESCs, displayed flatten morphology indicative of ESC differentiation (Figure 4D). Consistently, expression of Filia, but not Filia340, in FK ESCs restored their hypersensitivity to DNA damage (Figure 4D). Thus, Filia plays a critical role in ensuring ESCs' hypersensitivity to DNA damage, and this depends on its C-terminus.

Phosphorylation of Serine (S) 349 is required for Filia function in DNA damage repair

Our data suggest that Filia, akin to p53 in somatic cells, functions in two opposite aspects of DDR in ESCs. It is required for DNA repair that enables cells to survive the damage. It is also essential for damaged cells to undergo differentiation and apoptosis. Phosphorylation often correlates with a change in protein functions. There is evidence to suggest that S349 residue on Filia is subjected to phosphorylation in response to DNA damage (Pines et al., 2011). Therefore, we investigated if S349 was indeed phosphorylated and whether this modification played a role in modulating Filia's functions. Accordingly, we mutated S349 into alanine (S349A) that can no longer be phosphorylated and stably expressed this mutant in FK ESCs (FK-S349A) (Figure S4A). FiliaS349A protein rescued the observed defects in DDR signaling (Figure S4B), intra-S and G2/M cell cycle checkpoints in FK ESCs (Figures S4C and S4D). However, it failed to restore DNA repair as demonstrated by the comet assay under normal and etoposide treated conditions (Figures 5A and 5B). Consistently, FiliaS349A failed to rescue Rad51 recruitment to damage sites (Figure S4E). As a result, FK-S349A ESCs were more sensitive to DNA damage and prone to undergo apoptosis compared with WT ESCs (Figures 5C-5E). Consequently, these cells could not be maintained in culture for more than 12 passages. To further validate the phosphorylation of S349, we generated a polyclonal antibody that specifically recognizes the phosphorylated Filia at S349 (p-Filia(S349)). Immunoblotting revealed a specific ~70KD band, which displayed increasing intensity in response to DNA damage, in WT, but not FK-S349A ESCs (Figure 5F). Together, these data indicate that S349 residue of Filia is phosphorylated in response to DNA damage and this modification is essential for Filia's function in DNA damage repair. Moreover, S349 is not a substrate of ATM, as suppressing ATM kinase activity by KU55933 does not affect S349 phosphorylation (Figure S3H).

To further explore the biological significance of S349 phosphorylation, we substituted serine with aspartic acid (D) to mimic its phosphorylation and stably expressed FiliaS349D in FK ESCs (FK-S349D rescue cells, Figure S4A). FiliaS349D restored the DDR signaling (Figure S4B) and cell cycle checkpoints (Figures S4C and S4D), but failed to rescue the damage repair (Figures 5A, 5B, and S4E). Surprisingly, S349D severely impaired Filia's ability in regulating apoptosis, which correlates with a hyper-toleration of FK-S349D cells to DNA damage compared to either WT or FK ESCs (Figures 5D, 5G, and 5H). Together, these data support the notion that S349 phosphorylation is required for DNA repair, whereas non-phosphorylation status might be critical for Filia's pro-apoptotic function.

Filia dynamically translocates to different sub-cellular sites in response to DNA damage

We next examined Filia's sub-cellular localization under normal and DNA damage conditions. 3xFLAG tagged Filia, Filia340, FiliaS349A, or FiliaS349D were stably expressed in FK ESCs. Immunostaining revealed that Filia is primarily located in cytoplasm regardless of cell cycle or genotoxic damage (Figure S5A). Centrosomes are considered as command centers for cellular control and are known to integrate cell cycle regulation and DNA repair (Doxsey et al., 2005; Loffler et al., 2006; Shimada and Komatsu, 2009). To examine whether Filia is localized on centrosomes, we extracted free cytosolic Filia and co-stained FLAG-tagged Filia with pericentrin and γ-tubulin, two integral components of pericentriolar material. Filia accumulated on centrosomes at interphase and mitotic phase (Figures 6A and S5B) under normal condition. DNA damage did not enhance the centrosomal accumulation of Filia (Figure S5B), suggesting that this localization was constitutive. Centrosomal localization was also observed for Filia340, FiliaS349A and FiliaS349D (Figure S5C). However, p-Filia(S349) did not localize to centrosome (Figure 6A). Interestingly, Chk2 resides on centrosomes of mESCs (Hong and Stambrook, 2004). p-ATM was also detected on centrosomes of WT and rescue ESCs, but not FK cells (Figure S5D). These findings suggest that centrosome-localized Filia may play a role in regulating ATM, Chk2 and cell cycle checkpoints.

The constitutive centrosomal localization of Filia implied a role in regulating centrosome integrity. Centrosomes in FK ESCs were abnormal (Figure 1J). This could be a consequence of DNA damage (Bourke et al., 2007; Loffler et al., 2013). To exclude this possibility, we examined the centrosome integrity of FK-Filia, FK-Filia340, FK-S349A and FK-S349D rescue ESCs. Notably, all examined ESCs displayed grossly normal centrosomes and spindle assembly (Figure S5E), despite that sustained DNA damage was observed in FK-S349A and FK-S349D ESCs (Figure 5B). This observation excluded the causal relationship of DNA damage and centrosome defects observed in FK ESCs. Rather, it suggests that Filia itself plays a direct role in maintaining centrosome integrity. Indeed, co-immunoprecipitation combined with mass spectrometry identified Numa, a critical regulator of spindle pore integrity (Silk et al., 2009; Zeng, 2000), as an interacting protein of Filia on centrosome (Figure 6B). C-terminus of Filia (aa341-440) contributes to this interaction because there was an impairment of interaction between Numa and Filia340 compared with full-length Filia (Figure 6B).

In addition to the cytoplasmic distribution, there was a small amount of Filia in nuclei as determined by immunostaining and nucleus fractionation (Figures S5A and 6C). To confirm the presence of nuclear Filia, we treated the WT and FLAG-Filia rescued ESCs with leptomycin B (LPB) to inhibit nuclear protein export (Alpatov et al., 2014; Tamanini et al., 1999). Nuclear localization of Filia was clearly visible after LPB treatment (Figure 6D). Under normal condition, Filia was diffused in nucleus. DNA damage evoked increase in both the abundance of nuclear Filia and its translocation to the damage sites as labeled by γH2AX (Figures 6C and 6D). Intriguingly, S to A mutation (FLAG-S349A cells) prevented the entry of Filia into nuclei regardless of DNA damage (Figures 6C and 6D), indicating that Filia S349 phosphorylation is required for its nuclei localization. Consistently, p-Filia(S349) was exclusively stained for nuclei of WT ESCs under normal condition and co-localized

with γH2AX upon DNA damage (Figures 6C and 6E). Filia340 showed similar nuclear distribution as full Filia (Figures S6A and S6B), which correlates with its ability to restore DNA repair. Intriguingly, FiliaS349D protein exhibited proper nuclei localization (Figures S6A and S6B), despite its inability to repair DNA damage.

We next determined if Filia translocated to mitochondria upon apoptosis induction. Under normal condition, basal level of Filia was detected in mitochondria as assayed by immunostaining and mitochondria fractionation. Apoptosis induction with etoposide triggered a robust translocation of Filia into mitochondria (Figures 6F and 6G). Filia's localization to mitochondria was compromised in Filia340 and FiliaS349D, while was enhanced in FiliaS349A (Figures 6F and S6C). These results support the notion that localization of Filia in mitochondria is necessary for its apoptosis-promoting role. They also implied that mitochondria translocation of Filia requires its C-terminus and S349 at non-phosphorylated status. Consistently, p-Filia(S349) was not detected in mitochondria (Figure 6G).

Filia interacts with PARP1 and stimulates its enzymatic activity in DDR

To further explore the mechanistic basis by which Filia regulates DDR, we performed co-immunoprecipitation combined with mass spectrometry analysis. We identified PARP1 as one of the Filia's interaction proteins (Figure 7A). PARP1 catalyzes the poly(ADP-ribosyl)ation (PARylation) of its target proteins and plays a key role in early DDR (De Vos et al., 2012; Krishnakumar and Kraus, 2010). The interaction between PARP1 and Filia was validated in NIH/3T3 cells ectopically expressing Filia (Figure 7B). Interaction between PARP1 and Filia is regulated, and it was enhanced by DNA damage (Figure 7A). Moreover, the interaction did not require the C-terminus of Filia because Filia340 pulled down PARP1 efficiently (Figure 7A). Although these two proteins interact, there was no obvious co-localization between them. PARP1 was predominantly distributed in nuclei (Figure S7A), whereas the majority of Filia was detected in cytoplasm (Figure S5A). Unlike Filia (Figure 6D), PARP1 nuclear foci were typically not co-localized with γH2AX foci induced by DNA damage (Figure S7A). These observations support the notion that the interaction between Filia and PARP1 is transient.

To determine whether PARP1 is responsible for PARylation in ESCs, we inhibited PARP1 enzymatic activity using a specific inhibitor AG14361 (Calabrese et al., 2004) (Figure S7B). Time-course analysis revealed that inhibition of PARP1 completely abolished PARylation (Figure S7C), indicating that PARP1 plays a major role in regulating PARylation in ESCs. We next examined if Filia regulates PARP1 activity by comparing PAR levels between WT and FK ESCs. In WT ESCs, PAR levels increased after etoposide treatment, whereas significantly lower PAR was detected in untreated or etoposide treated FK cells examined at 4 and 8 hr (Figure 7C). Thus, Filia is necessary for robust PARP1 activation in response to DNA damage. Intriguingly, PARP1-dependent but Filia-independent PAR was elevated at 12 hr in FK cells (Figures 7C and S7C). The Filia-independent PAR displayed abnormal accumulation at nucleolus (Figure S7D), which is known as a storage site for PAR in DDR (Mortusewicz et al., 2007). Re-expression of Filia, Filia340, FiliaS349A or FiliaS349D in FK ESCs efficiently restored PARP1 activity and the PAR levels (Figure 7C). Importantly,

differentiating ESCs and somatic cells have much less PAR compared with undifferentiated ESCs (Figures S7E and S7F). Ectopic expression of Filia and its variants in NIH/3T3 cells significantly increased the PAR levels (Figure 7D). The majority of PAR was detected in nucleus of ESCs expressing Filia, Filia340, and FiliaS349D, whereas PAR was predominantly accumulated in cytoplasm of FK-S349A rescue cells (Figure S7G). This is consistent with the cytoplasmic distribution of FiliaS349A. Notably, localization of Filia to DNA damage site did not rely on PAR modification (Figure S7H).

To determine if PARP1 plays a role in mediating Filia function in DDR, we inhibited PARP1 activity using AG14361 and examined its effects on ATM and Chk2 activation, cell cycle checkpoints, DNA damage repair and apoptosis. Inhibition of PARP1 significantly attenuated but not completely blocked ATM and Chk2 activation (Figure 7E). Notably, PARP1 inhibition caused the same extent of defects in cell cycle checkpoints and DNA damage repair as Filia knockout (Figures 7F-7H). Consistently, ectopic expression of Filia in NIH/3T3 cells not only increased PAR levels (Figure 7D), but also enhanced DNA damage repair in these cells (Figure 7I). However, DNA damage induced apoptosis was not impaired by PARP1 inhibition (Figure 7E). This suggests that PARP1 activity is not necessary for apoptosis induction. Together, these data support the notion that Filia interacts with PARP1 and activates its enzymatic activity in response to DNA damage, which contributes to the observed roles of Filia in regulating DDR signaling, cell cycle checkpoints and DNA damage repair.

Discussion

Compared to somatic cells, PSCs have superior competence and unique strategies to cope with DNA damage in order to maintain genomic integrity. In addition to using common proteins found in somatic cells with alternative strategies (Dumitru et al., 2012), PSCs possess unique proteins to safeguard their genome integrity (Zalzman et al., 2010). However, little is known regarding the PSC-specific factors in regulating genomic stability. Here, we identify an ESC-specific protein, Filia, as a powerful regulator of genomic stability. Through its coordinated cytoplasmic and nuclear functions, Filia regulates centrosome integrity and DDR at multiple levels. These include DDR signal transduction, cell cycle checkpoints, DNA damage repair and apoptosis. Thus, Filia depletion not only causes robust genomic instability, but also impedes elimination of damaged cells by ESC differentiation or apoptosis. This, in turn, increases the risk of transformation and tumorigenesis in ESCs. In somatic cells, p53 plays similar dual regulatory roles in DDR (Green and Kroemer, 2009). However, these well-established roles of p53 are not evident in mESC (Zhao and Xu, 2010). We hypothesize that PSCs utilize specific factors such as Filia to safeguard their genome integrity. Of note, reprogramming somatic cells into induced PSCs (iPSCs) is characterized by large variation in *Filia* (*Ecat1*) expression (Aoi et al., 2008; Kaji et al., 2009; Takahashi and Yamanaka, 2006). This suggests that *Filia* expression is a potential molecular marker that correlates with iPSC quality.

Filia carries out its multiple functions through different mechanisms. On one hand, Filia is dynamically translocated to centrosomes, DNA damage sites and mitochondria to execute its regulations on DDR independent on PARP1. On the other hand, Filia physically interacts

with PARP1 and stimulates PARP1's enzymatic activity to regulate DDR. PARP1 plays a key role in regulating DDR and genomic integrity (Krishnakumar and Kraus, 2010; Luo and Kraus, 2012). For instance, PARP1 or PAR-deficient cells are compromised in ATM activation, DNA damage signaling, cell cycle checkpoints, and DNA repair (Aguilar-Quesada et al., 2007; Haince et al., 2007; Min et al., 2013). Knockout mice for *Parp1* or *Parp2* are hypersensitive to DNA-damaging agents and display increased genomic instability after genotoxic stress (Hassa et al., 2006). More than 100 PARylation targets were identified in DDR, including PARP1 itself, histones, CDK2, kinases, and damage repair proteins (Jungmichel et al., 2013). Filia is uniquely expressed in ESCs, which correlates with high PAR levels and superior ability to maintain genomic stability in these cells. Thus, these findings support a model whereby activation of PARP1 by Filia contributes to the observed phenotypes in ATM and Chk2 activation, cell cycle checkpoints and DNA damage repair in FK ESCs.

Filia and PARP1 do not apparently co-localize. In addition, FiliaS349A is able to stimulate PARP1 activity despite its restrictive cytoplasmic distribution. These findings suggest that the dynamic interaction between PARP1 and Filia and activation of PARP1 by Filia occur in both cytoplasm and nuclei. PAR regulates protein's sub-cellular redistribution, this provides a possible explanation for the presence of PAR and p-ATM in nuclei of FiliaS349A rescued ESCs.

Compared with Filia knockout, PARP1 inhibition caused a less obvious defect in ATM and Chk2 activation in response to DNA damage. Filia340 failed to rescue the defects in cell cycle checkpoints despite its ability to activate PARP1. These findings suggest that Filia also regulates ATM-Chk2 activation and cell cycle checkpoints in PARP1-independent manner. ATM activation exhibits two phases and the first phase is not overtly affected by Filia knockout. This might be due to the presence of basal levels of PAR in Filia knockout cells, which accounts for the first phase of ATM activation. Centrosomes are known to integrate many regulatory factors that control cell cycle progression and DDR. Cell cycle regulators such as Cdk-cyclin complex (Bailly et al., 1992; Hinchcliffe et al., 1999), Chk1 (Kramer et al., 2004; Zhang et al., 2007) and Chk2 (Golan et al., 2010; Hong and Stambrook, 2004; Zhang et al., 2007) are present on centrosomes. Furthermore, DDR regulators such as ATM, ATR and DNA-PK have also been shown to reside on centrosomes (Zhang et al., 2007). Consistently, we observed the localization of p-ATM on centrosomes of ESCs. However, p-ATM was absent from centrosomes when Filia was depleted. Re-expression of Filia340, FiliaS349A or FiliaS349D, all of which localize on centrosomes, restored second wave of ATM activation. Therefore, the centrosomal localized Filia may coordinate the control of DDR signaling, cell cycle checkpoints and centrosome integrity.

FiliaS349A or FiliaS349D rescued ESCs fail to repair DNA damage despite that the PARP1 activity and PAR levels are normal. This is consistent with the idea that Filia also regulates DNA damage repair in a PARP1-independent manner. The phosphorylation of S349 at C-terminus is necessary for Filia's nuclei localization. However, without the C-terminus, Filia340 is localized to nuclei (Figure S6B). Notably, bioinformatics analysis indicates that Filia340 (aa1-340) contains potential nuclear localization signal (NLS). Thus, it is possible that the phosphorylation of S349 regulates NLS function. Specifically, non-phosphorylated

S349 suppresses NLS function, whereas phosphorylation of S349 or simply removing C-terminus activates NLS function. Upon LPB treatment, nuclear Filia is diffusive in the absence of DNA damage. DNA damage triggers the translocation of Filia to DNA damage sites, indicating that Filia's entry into nuclei and its translocation to DNA damage sites are regulated separately. PARylation plays a critical role in recruiting DNA repair proteins to damage sites (Krishnakumar and Kraus, 2010; Tallis et al., 2014). However, localization of Filia into DNA damage sites is regulated neither by PARylation, nor by interaction with PARP1.

In summary, our data demonstrate that Filia functions as the first of its kind ESC-specific regulator of genome integrity. These data support the notion that ESCs employ distinct mechanisms and utilize specific factors such as Filia to safeguard their genomic integrity. They also suggest that Filia expression level is a potential biomarker for the quality of iPS cells with regard to genomic stability and transformation potential.

Experimental procedures

Derivation and culture of mouse ESCs

All experimental procedures and animal care were performed according to the protocols approved by the Ethics Committee of the Kunming Institute of Zoology, Chinese Academy of Sciences. MEF preparation, ESC derivation and culture were performed in standard ways (Evans and Kaufman, 1981). ESC Genotyping was performed as described (Zheng and Dean, 2009).

Cytogenetic analysis and telomere fluorescent in situ hybridization (T-FISH)

Metaphase chromosome spreads were prepared and stained with Giemsa solution or DAPI. For T-FISH, metaphase spreads were prepared. DNA was denatured and hybridized with Peptide-nucleic acid (PNA) telomere probes (Tel G - Alexa 488, PANAGENE, F1010-5) (Lansdorp et al., 1996). DNA was counterstained with DAPI.

Immunoblotting and immunofluorescent staining

Immunoblotting and immunofluorescent staining were performed according to the standard procedures (Narva et al., 2012). The primary antibodies were listed in Table S1. Rabbit polyclonal antibodies against Filia and p-Filia(S349) were generated by Abmart.

Cell cycle checkpoints analysis

ESCs were treated with hydroxyl urea to induce replication stress and S-phase arrest was evaluated by EdU pulse-chase incorporation assay (Buck et al., 2008). G2/M checkpoint was examined by standard methods after treating ESCs with etoposide (Greer Card et al., 2010).

Alkaline comet assay and clonal competition assay

Alkaline comet assay was performed according to the standard procedure (Tice et al., 2000). Comets were analyzed using CASP comet assay software (Andor Technology) and 100 cells were calculated in each sample. Two types of ESCs, of which one was labeled with GFP expression, were mixed in same number. The mixed ESCs were subjected to the same

genotoxic stress and co-culture. Colony growth was monitored daily and 200 clones were examined at each time point.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Jurrien Dean at the National Institutes of Health, USA for kindly providing *Filia* mutant mice; Weijian Sang, Rui Bi, and Zhengyuan Xie for technical support in making lentival expression vectors; The protein mass spectrometry center in Shanghai Institute of Materia Medica, CAS for performing the mass spectrometry analysis; Dr. Jurrien Dean at NIH and Dr. Jiali Li at Kunming Institute of Zoology, CAS for careful reading and editing the paper. This work is supported by the Chinese NSFC grant to P.Z. (31171424), the CAS "Strategic Priority Research Program" (XDA01010203); and the talent grant from Yunnan province. R.Z. is supported by NIH/NCI R01CA160331 and Cancer Center Core grant CA010815.

References

Aguilar-Quesada R, Munoz-Gamez JA, Martin-Oliva D, Peralta A, Valenzuela MT, Matinez-Romero R, Quiles-Perez R, Menissier-de Murcia J, de Murcia G, Ruiz de Almodovar M, et al. Interaction between ATM and PARP-1 in response to DNA damage and sensitization of ATM deficient cells through PARP inhibition. BMC Mol Biol. 2007; 8:29. [PubMed: 17459151]

Alpatov R, Lesch BJ, Nakamoto-Kinoshita M, Blanco A, Chen S, Stutzer A, Armache KJ, Simon MD, Xu C, Ali M, et al. A chromatin-dependent role of the fragile X mental retardation protein FMRP in the DNA damage response. Cell. 2014; 157:869–881. [PubMed: 24813610]

Aoi T, Yae K, Nakagawa M, Ichisaka T, Okita K, Takahashi K, Chiba T, Yamanaka S. Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science. 2008; 321:699–702. [PubMed: 18276851]

Bailly E, Pines J, Hunter T, Bornens M. Cytoplasmic accumulation of cyclin B1 in human cells: association with a detergent-resistant compartment and with the centrosome. J Cell Sci. 1992; 101:529–545. Pt 3. [PubMed: 1387877]

Bartkova J, Horejsi Z, Koed K, Kramer A, Tort F, Zieger K, Guldberg P, Sehested M, Nesland JM, Lukas C, et al. DNA damage response as a candidate anti-cancer barrier in early human tumorigenesis. Nature. 2005; 434:864–870. [PubMed: 15829956]

Batchelor E, Mock CS, Bhan I, Loewer A, Lahav G. Recurrent initiation: a mechanism for triggering p53 pulses in response to DNA damage. Mol Cell. 2008; 30:277–289. [PubMed: 18471974]

Behrens A, van Deursen JM, Rudolph KL, Schumacher B. Impact of genomic damage and ageing on stem cell function. Nat Cell Biol. 2014; 16:201–207. [PubMed: 24576896]

Bourke E, Dodson H, Merdes A, Cuffe L, Zachos G, Walker M, Gillespie D, Morrison CG. DNA damage induces Chk1-dependent centrosome amplification. EMBO Rep. 2007; 8:603–609. [PubMed: 17468739]

Branzei D, Foiani M. Regulation of DNA repair throughout the cell cycle. Nat Rev Mol Cell Biol. 2008; 9:297–308. [PubMed: 18285803]

Buck SB, Bradford J, Gee KR, Agnew BJ, Clarke ST, Salic A. Detection of S-phase cell cycle progression using 5-ethynyl-2'-deoxyuridine incorporation with click chemistry, an alternative to using 5-bromo-2'-deoxyuridine antibodies. Biotechniques. 2008; 44:927–929. [PubMed: 18533904]

Calabrese CR, Almassy R, Barton S, Batey MA, Calvert AH, Canan-Koch S, Durkacz BW, Hostomsky Z, Kumpf RA, Kyle S, et al. Anticancer chemosensitization and radiosensitization by the novel poly(ADP-ribose) polymerase-1 inhibitor AG14361. J Natl Cancer Inst. 2004; 96:56–67. [PubMed: 14709739]

Davis AJ, Chen BP, Chen DJ. DNA-PK: a dynamic enzyme in a versatile DSB repair pathway. DNA Repair (Amst). 2014; 17:21–29. [PubMed: 24680878]

De Vos M, Schreiber V, Dantzer F. The diverse roles and clinical relevance of PARPs in DNA damage repair: current state of the art. Biochem Pharmacol. 2012; 84:137–146. [PubMed: 22469522]

Doxsey S, Zimmerman W, Mikule K. Centrosome control of the cell cycle. Trends Cell Biol. 2005; 15:303–311. [PubMed: 15953548]

Dumitru R, Gama V, Fagan BM, Bower JJ, Swahari V, Pevny LH, Deshmukh M. Human embryonic stem cells have constitutively active Bax at the Golgi and are primed to undergo rapid apoptosis. Mol Cell. 2012; 46:573–583. [PubMed: 22560721]

Evans MJ, Kaufman MH. Establishment in culture of pluripotential cells from mouse embryos. Nature. 1981; 292:154–156. [PubMed: 7242681]

Farber-Katz SE, Dippold HC, Buschman MD, Peterman MC, Xing M, Noakes CJ, Tat J, Ng MM, Rahajeng J, Cowan DM, et al. DNA damage triggers Golgi dispersal via DNA-PK and GOLPH3. Cell. 2014; 156:413–427. [PubMed: 24485452]

Golan A, Pick E, Tsvetkov L, Nadler Y, Kluger H, Stern DF. Centrosomal Chk2 in DNA damage responses and cell cycle progression. Cell Cycle. 2010; 9:2647–2656. [PubMed: 20581449]

Green DR, Kroemer G. Cytoplasmic functions of the tumour suppressor p53. Nature. 2009; 458:1127–1130. [PubMed: 19407794]

Greer Card DA, Sierant ML, Davey S. Rad9A is required for G2 decatenation checkpoint and to prevent endoreduplication in response to topoisomerase II inhibition. J Biol Chem. 2010; 285:15653–15661. [PubMed: 20305300]

Haince JF, Kozlov S, Dawson VL, Dawson TM, Hendzel MJ, Lavin MF, Poirier GG. Ataxia telangiectasia mutated (ATM) signaling network is modulated by a novel poly(ADP-ribose)-dependent pathway in the early response to DNA-damaging agents. J Biol Chem. 2007; 282:16441–16453. [PubMed: 17428792]

Hassa PO, Haenni SS, Elser M, Hottiger MO. Nuclear ADP-ribosylation reactions in mammalian cells: where are we today and where are we going? Microbiol Mol Biol Rev. 2006; 70:789–829. [PubMed: 16959969]

Hinchcliffe EH, Li C, Thompson EA, Maller JL, Sluder G. Requirement of Cdk2-cyclin E activity for repeated centrosome reproduction in Xenopus egg extracts. Science. 1999; 283:851–854. [PubMed: 9933170]

Hirao A, Kong YY, Matsuoka S, Wakeham A, Ruland J, Yoshida H, Liu D, Elledge SJ, Mak TW. DNA damage-induced activation of p53 by the checkpoint kinase Chk2. Science. 2000; 287:1824–1827. [PubMed: 10710310]

Hong Y, Stambrook PJ. Restoration of an absent G1 arrest and protection from apoptosis in embryonic stem cells after ionizing radiation. Proc Natl Acad Sci U S A. 2004; 101:14443–14448. [PubMed: 15452351]

Inomata K, Aoto T, Binh NT, Okamoto N, Tanimura S, Wakayama T, Iseki S, Hara E, Masunaga T, Shimizu H, et al. Genotoxic stress abrogates renewal of melanocyte stem cells by triggering their differentiation. Cell. 2009; 137:1088–1099. [PubMed: 19524511]

Jackson SP, Bartek J. The DNA-damage response in human biology and disease. Nature. 2009; 461:1071–1078. [PubMed: 19847258]

Jungmichel S, Rosenthal F, Altmeyer M, Lukas J, Hottiger MO, Nielsen ML. Proteome-wide identification of poly(ADP-Ribosyl)ation targets in different genotoxic stress responses. Mol Cell. 2013; 52:272–285. [PubMed: 24055347]

Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P, Woltjen K. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. Nature. 2009; 458:771–775. [PubMed: 19252477]

Kramer A, Mailand N, Lukas C, Syljuasen RG, Wilkinson CJ, Nigg EA, Bartek J, Lukas J. Centrosome-associated Chk1 prevents premature activation of cyclin-B-Cdk1 kinase. Nat Cell Biol. 2004; 6:884–891. [PubMed: 15311285]

Krishnakumar R, Kraus WL. The PARP side of the nucleus: molecular actions, physiological outcomes, and clinical targets. Mol Cell. 2010; 39:8–24. [PubMed: 20603072]

Lansdorp PM, Verwoerd NP, van de Rijke FM, Dragowska V, Little MT, Dirks RW, Raap AK, Tanke HJ. Heterogeneity in telomere length of human chromosomes. Hum Mol Genet. 1996; 5:685–691. [PubMed: 8733138]

Li M, He Y, Dubois W, Wu X, Shi J, Huang J. Distinct regulatory mechanisms and functions for p53-activated and p53-repressed DNA damage response genes in embryonic stem cells. Mol Cell. 2012; 46:30–42. [PubMed: 22387025]

Liang Y, Lin SY, Brunicardi FC, Goss J, Li K. DNA damage response pathways in tumor suppression and cancer treatment. World J Surg. 2009; 33:661–666. [PubMed: 19034564]

Lin T, Chao C, Saito S, Mazur SJ, Murphy ME, Appella E, Xu Y. p53 induces differentiation of mouse embryonic stem cells by suppressing Nanog expression. Nat Cell Biol. 2005; 7:165–171. [PubMed: 15619621]

Liu JC, Guan X, Ryan JA, Rivera AG, Mock C, Agarwal V, Letai A, Lerou PH, Lahav G. High mitochondrial priming sensitizes hESCs to DNA-damage-induced apoptosis. Cell Stem Cell. 2013; 13:483–491. [PubMed: 23954752]

Liu Q, Guntuku S, Cui XS, Matsuoka S, Cortez D, Tamai K, Luo G, Carattini-Rivera S, DeMayo F, Bradley A, et al. Chk1 is an essential kinase that is regulated by Atr and required for the G(2)/M DNA damage checkpoint. Genes Dev. 2000; 14:1448–1459. [PubMed: 10859164]

Loffler H, Fechter A, Liu FY, Poppelreuther S, Kramer A. DNA damage-induced centrosome amplification occurs via excessive formation of centriolar satellites. Oncogene. 2013; 32:2963–2972. [PubMed: 22824794]

Loffler H, Lukas J, Bartek J, Kramer A. Structure meets function--centrosomes, genome maintenance and the DNA damage response. Exp Cell Res. 2006; 312:2633–2640. [PubMed: 16854412]

Luo X, Kraus WL. On PAR with PARP: cellular stress signaling through poly(ADP-ribose) and PARP-1. Genes Dev. 2012; 26:417–432. [PubMed: 22391446]

Min W, Bruhn C, Grigaravicius P, Zhou ZW, Li F, Kruger A, Siddeek B, Greulich KO, Popp O, Meisezahl C, et al. Poly(ADP-ribose) binding to Chk1 at stalled replication forks is required for S-phase checkpoint activation. Nat Commun. 2013; 4:2993. [PubMed: 24356582]

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell. 2003; 113:631–642. [PubMed: 12787504]

Momcilovic O, Choi S, Varum S, Bakkenist C, Schatten G, Navara C. Ionizing radiation induces ataxia telangiectasia mutated-dependent checkpoint signaling and G(2) but not G(1) cell cycle arrest in pluripotent human embryonic stem cells. Stem Cells. 2009; 27:1822–1835. [PubMed: 19544417]

Momcilovic O, Navara C, Schatten G. Cell cycle adaptations and maintenance of genomic integrity in embryonic stem cells and induced pluripotent stem cells. Results Probl Cell Differ. 2011; 53:415–458. [PubMed: 21630155]

Mortusewicz O, Ame JC, Schreiber V, Leonhardt H. Feedback-regulated poly(ADP-ribosyl)ation by PARP-1 is required for rapid response to DNA damage in living cells. Nucleic Acids Res. 2007; 35:7665–7675. [PubMed: 17982172]

Nagy Z, Soutoglou E. DNA repair: easy to visualize, difficult to elucidate. Trends Cell Biol. 2009; 19:617–629. [PubMed: 19819145]

Narva E, Rahkonen N, Emani MR, Lund R, Pursiheimo JP, Nasti J, Autio R, Rasool O, Denessiouk K, Lahdesmaki H, et al. RNA-binding protein L1TD1 interacts with LIN28 via RNA and is required for human embryonic stem cell self-renewal and cancer cell proliferation. Stem Cells. 2012; 30:452–460. [PubMed: 22162396]

Ohsugi M, Zheng P, Baibakov B, Li L, Dean J. Maternally derived FILIA-MATER complex localizes asymmetrically in cleavage-stage mouse embryos. Development. 2008; 135:259–269. [PubMed: 18057100]

Pines A, Kelstrup CD, Vrouwe MG, Puigvert JC, Typas D, Misovic B, de Groot A, von Stechow L, van de Water B, Danen EH, et al. Global phosphoproteome profiling reveals unanticipated networks responsive to cisplatin treatment of embryonic stem cells. Mol Cell Biol. 2011; 31:4964–4977. [PubMed: 22006019]

Rass U, Ahel I, West SC. Defective DNA repair and neurodegenerative disease. Cell. 2007; 130:991–1004. [PubMed: 17889645]

Shimada M, Komatsu K. Emerging connection between centrosome and DNA repair machinery. J Radiat Res. 2009; 50:295–301. [PubMed: 19542690]

Silk AD, Holland AJ, Cleveland DW. Requirements for NuMA in maintenance and establishment of mammalian spindle poles. J Cell Biol. 2009; 184:677–690. [PubMed: 19255246]

Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006; 126:663–676. [PubMed: 16904174]

Tallis M, Morra R, Barkauskaite E, Ahel I. Poly(ADP-ribosyl)ation in regulation of chromatin structure and the DNA damage response. Chromosoma. 2014; 123:79–90. [PubMed: 24162931]

Tamanini F, Bontekoe C, Bakker CE, van Unen L, Anar B, Willemsen R, Yoshida M, Galjaard H, Oostra BA, Hoogeveen AT. Different targets for the fragile X-related proteins revealed by their distinct nuclear localizations. Hum Mol Genet. 1999; 8:863–869. [PubMed: 10196376]

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ Mol Mutagen. 2000; 35:206–221. [PubMed: 10737956]

Tichy ED, Pillai R, Deng L, Liang L, Tischfield J, Schwemberger SJ, Babcock GF, Stambrook PJ. Mouse embryonic stem cells, but not somatic cells, predominantly use homologous recombination to repair double-strand DNA breaks. Stem Cells Dev. 2010; 19:1699–1711. [PubMed: 20446816]

van der Laan S, Tsanov N, Crozet C, Maiorano D. High Dub3 expression in mouse ESCs couples the G1/S checkpoint to pluripotency. Mol Cell. 2013; 52:366–379. [PubMed: 24207026]

Wyles SP, Brandt EB, Nelson TJ. Stem cells: the pursuit of genomic stability. Int J Mol Sci. 2014; 15:20948–20967. [PubMed: 25405730]

Yamamoto K, Wang Y, Jiang W, Liu X, Dubois RL, Lin CS, Ludwig T, Bakkenist CJ, Zha S. Kinase-dead ATM protein causes genomic instability and early embryonic lethality in mice. J Cell Biol. 2012; 198:305–313. [PubMed: 22869596]

Zalzman M, Falco G, Sharova LV, Nishiyama A, Thomas M, Lee SL, Stagg CA, Hoang HG, Yang HT, Indig FE, et al. Zscan4 regulates telomere elongation and genomic stability in ES cells. Nature. 2010; 464:858–863. [PubMed: 20336070]

Zeng C. NuMA: a nuclear protein involved in mitotic centrosome function. Microsc Res Tech. 2000; 49:467–477. [PubMed: 10842374]

Zhang S, Hemmerich P, Grosse F. Centrosomal localization of DNA damage checkpoint proteins. J Cell Biochem. 2007; 101:451–465. [PubMed: 17171639]

Zhao T, Xu Y. p53 and stem cells: new developments and new concerns. Trends Cell Biol. 2010; 20:170–175. [PubMed: 20061153]

Zheng P, Dean J. Role of Filia, a maternal effect gene, in maintaining euploidy during cleavage-stage mouse embryogenesis. Proc Natl Acad Sci U S A. 2009; 106:7473–7478. [PubMed: 19376971]

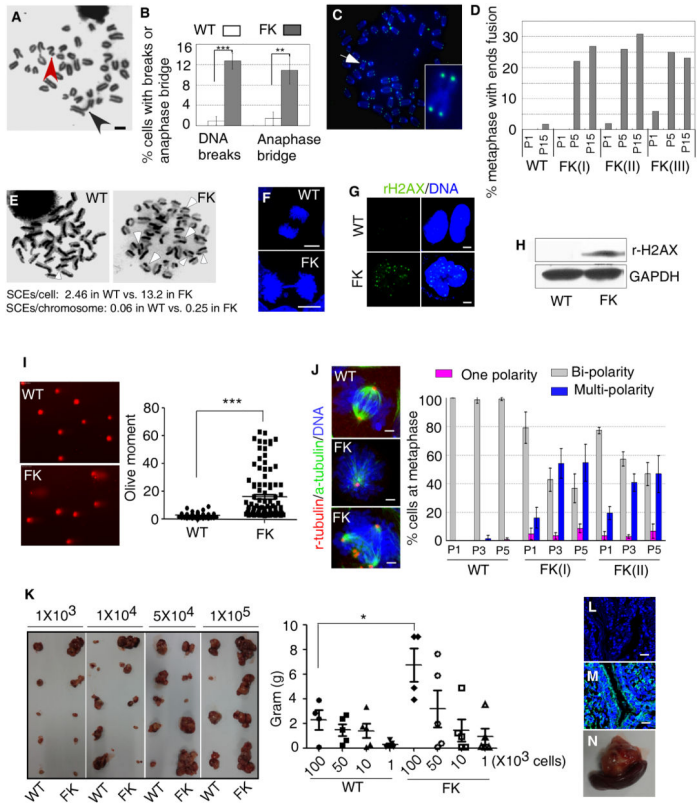

Figure 1. Filia maintains genomic stability and prevents malignant transformation of ESCs

(**A**) Metaphase chromosome spread of FK ESC. Arrow heads indicate chromosome break (red) and chromosome-end fusion (black). (**B**) Frequencies of chromosome breaks and anaphase brideges in WT and FK ESCs. >200 cells were examined in each sample. (**C**) Chromosome-end fusion detected by telomere-FISH. (**D**) Frequencies of metaphase with chromosome-end fusion in WT and FK ESCs. >100 metaphase spreads were examined in each sample. (**E**) SCE (arrow heads) in WT and FK ESCs. 50 cells were examined in each group. (**F**) Anaphase brideges in FK ESCs. (**G**) γH2AX foci in WT and FK ESCs. (**H**) γH2AX accumulation in WT and FK cells detected by immunoblotting. (**I**) Comet assay showed FK ESCs had severe DNA damage. (**J**) Centrosomes in FK and WT ESCs at passages 3 and 5 (P3 and P5). >200 cells were examined in each of the indicated groups. (**K**) Teratomas formed from FK ESCs were bigger and weighed more than those from WT ESCs injected at different concentrations. (**L**) Teratoma tissue formed by FK ESCs that were GFP negative. (**M**) Teratoma tissue formed by GFP-labeled WT ESCs. (N) FK ESC formed tumors in pancreas. Scale bar, 10 μm. *, P<0.05; **, P<0.01; ***, P<0.001. See also Figures S1 and S2.

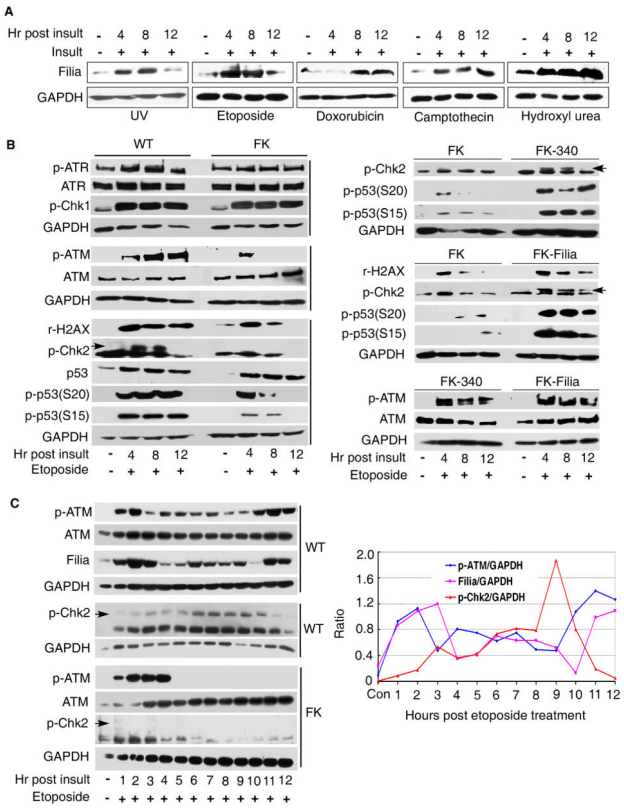

Figure 2. Filia regulates DNA damage signaling

(**A**) Filia protein expression was up-regulated by genotoxic insults. (**B**) The induction of γH2AX, p-ATM, p-Chk2, and p-p53 were compromised in FK ESCs, whereas p-ATR or p-Chk1 was not affected. Re-expression of Filia or Filia340 sucessfully rescued the defects. (**C**) Detailed time-course analysis of p-ATM, p-Chk2, and Filia expression in WT and FK ESCs treated with etoposide. Arrows indicate the p-Chk2. See also Figures S2 and S3.

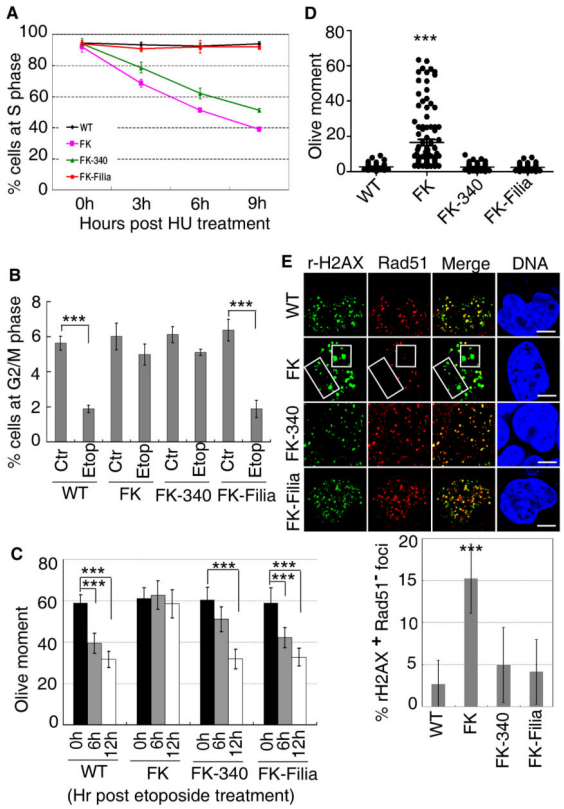

Figure 3. Filia is necessary for cell cycle checkpoints and DNA damage repair

(**A**) The S phase checkpoint was functional in WT and FK-Filia rescue ESCs, but failed in FK and FK-340 cells. (**B**) Similarly, FK ESCs lost G2 checkpoint which was restored in FK-Filia, but not FK-340 ESCs. (**C**) WT, FK-Filia and FK-340 ESCs could repair DNA damage induced by etoposide treatment, whereas FK ESCs could not. (**D**) DNA integrity assessment of WT, FK, FK-340 and FK-Filia ESCs by the comet assay showed persistent DNA breaks in FK ESCs. (**E**) Rad51 was recruited to DSB sites in WT, FK-340 and FK-Filia ESCs upon DNA damage. However, its recruitment was compromised in FK ESCs. Squares indicated the examples of $\gamma H2AX^{+}Rad51^{-}$ foci. 50 cells were examined in each sample. Scale bar, 10 μm. ***, P<0.001. See also Figure S3.

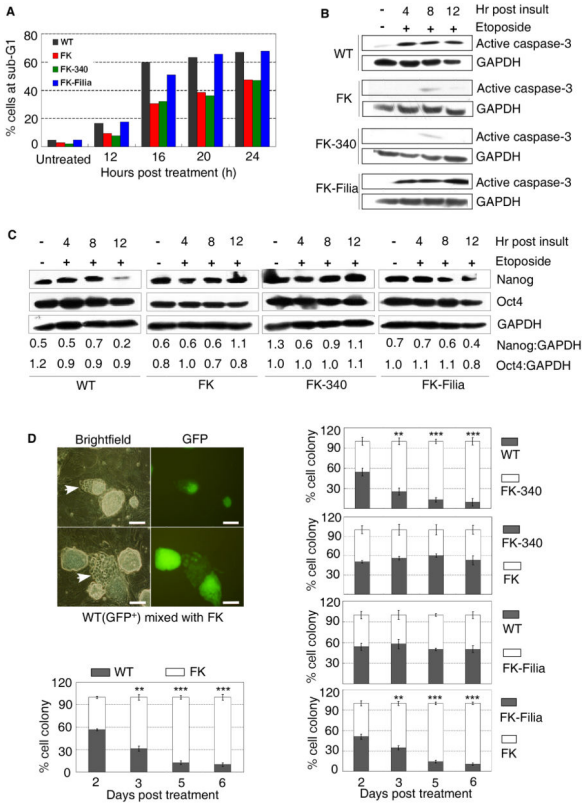

Figure 4. Filia is required for ESCs to undergo differentiation and apoptosis in response to DNA damage

(**A**) FACS analysis showed a higher proportion of WT and FK-Filia ESCs were at sub-G1 phase indicative of cell death after etoposide treatment. (**B**) WT and FK-Filia ESCs expressed more active caspase-3 than FK and FK-340 ESCs in response to etoposide treatment. (**C**) Nanog was down-regulated in WT and FK-Filia but not in FK or FK-340 ESCs after DNA damage. (**D**) Clonal competition assay revealed that FK ESCs were less sensitive to DNA damage than WT ESCs. Re-expression of Filia, but not Filia340, restored their sensitivity to DNA damage. Representative images of mixtures of WT (GFP$^+$) and FK ESC clones. Note that WT ESC clones (white arrow) initiated differentiation. **, P<0.01; ***, P<0.001.

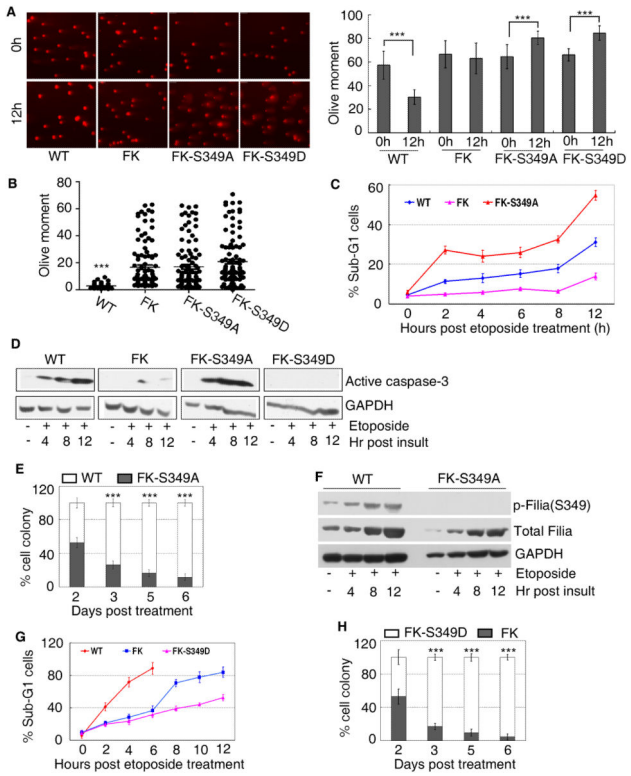

Figure 5. S349 is phosphorylated to modulate Filia's role in DNA damage repair

(**A**) After etoposide treatment, WT ESCs were more efficient to repair DNA damage than FK, FK-S349A and FK-S349D ESCs. (**B**) Consistently, untreated FK, FK-S349A and FK-S349D ESCs had severe DNA damage compared to WT ESCs. (**C**) Higher proportion of FK-S349A ESCs were dead compared to WT and FK ESCs after eoptoside treatment. (**D**) Consistently, FK-S349A ESCs expressed more active caspase-3 than WT, FK and FK-S349D ESCs. (**E**) Clonal competition assay confirmed that FK-S349A ESCs had superior sensitivity to DNA damage than WT cells. (**F**) Immunobloting with antibody specifically recognizing phosphorylated S349 in Filia validated this phosphorylation modification. Note that phosphorylation level was up-regulated by DNA damage. (**G**) Sub-G1 analysis indicated that FK-S349D ESCs were more resistant to apoptosis. (**H**) Clonal competition analysis further confirmed the higher tolerance of FK-S349D ESCs to DNA damage than FK ESCs. ***, P<0.001. See also Figures S3 and S4.

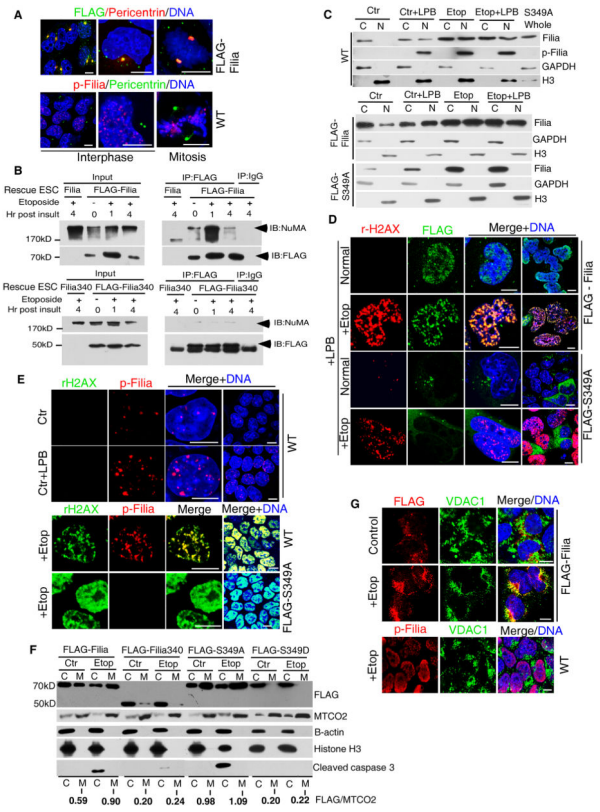

Figure 6. Filia constitutively localizes on centrosomes and DNA damage stimulates its translocation to damage sites or mitochondria

(**A**) Constitutive localization of Filia, but not p-Filia(S349), on centrosomes labeled by pericentrin. (**B**) Immunoprecipitation combined with immunoblotting confirmed the interaction of Filia or Filia340 with Numa. (**C**) Nucleus fractionation documented the presence of Filia in cytoplasm (C) and nucleus (N) of WT and FLAG-Filia rescued ESCs. However, FiliaS349A and p-Filia(S349) were exclusively detected in cytoplasm and nucleus, respectively. LPB as well as etoposide treatment increased nuclear accumulation of Filia and p-Filia. Whole lysates of FK-S349A ESCs were used as control. (**D**) Immunostaining revealed the nuclear localization of Filia, but not FiliaS349A proteins. Etoposide treatment stimulated the recruitment of Filia to DNA damage sites labeled with γH2AX. (**E**) In WT ESCs, p-Filia(S349) was detected in nucleus under normal and DNA damage conditions. Etoposide treatment increased the accumulation of p-Filia(S349) on DNA damage sites. FK-S349A cells serve as negative control. (**F**) Mitochondria fractionation revealed the localization of Filia and FiliaS349A on mitochondria (M) marked by MTCO2. Apoptosis induction by etoposide treatment evoked their accumulation on mitochondria. However, little Filia340 or FiliaS349D protein was detected on mitochondria under normal or DNA damage condition. C represented the fraction of whole cell lysates depleted of mitochondria. (**G**) Immunostaining confirmed the localization of Filia but not p-Filia(S349) on mitochondria marked with VDAC1. Scale bar, 10 μm. See also Figures S5 and S6.

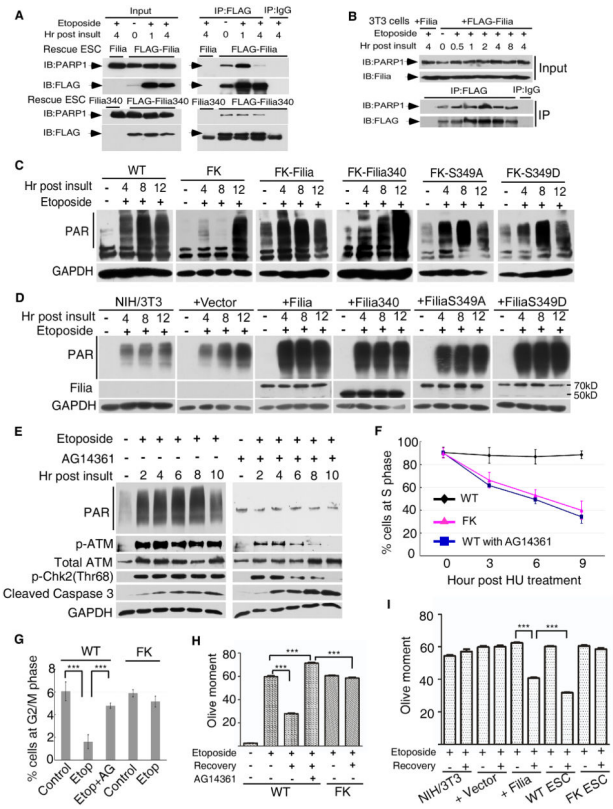

Figure 7. Filia interacts with PARP1 and stimulates its enzymatic activity which amplify Filia's roles in DDR

(**A**) Immunoprecipitation combined with immunoblotting confirmed the interaction of Filia or Filia340 with PARP1 in ESCs under normal or DNA damage condition. (**B**) Filia interacts with PARP1 in NIH/3T3 cells ectopically expressing Filia under normal or DNA damage condition. (**C**) FK ESCs had much lower PAR level than WT ESCs. However, re-expression of Filia or its variants restored the PAR level. (**D**) Similarly, ectopic expression of Filia or its variants in NIH/3T3 cells significantly increased the PAR level in response to DNA damage. (**E**) Inhibition of PARP1 activity by AG14361 significantly attenuated ATM and Chk2 activation. Consequently, cells with deficient PARP1 activity were prone to undergo apoptosis. Inhibition of PARP1 activity in ESCs abolished S-phase (**F**) and G2/M (**G**) cell cycle checkpoints, and compromised DNA damage repair (**H**). (**I**) Ectopic expression of Filia in NIH/3T3 cells significantly enhanced their DNA damage repair ability. NIH/3T3 cells, NIH/3T3 cells transfected with vectors, WT ESCs and FK ESCs were used as controls. In (H) and (I), cells were recovered for 12 hr. ***, P<0.001. See also Figure S7.