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RESEARCH ARTICLE

Nuclear α-catenin mediates the DNA damage response via β-catenin and nuclear actin

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ABSTRACT

α-Catenin is an F-actin-binding protein widely recognized for its role in cell–cell adhesion. However, a growing body of literature indicates that α-catenin is also a nuclear protein. In this study, we show that α-catenin is able to modulate the sensitivity of cells to DNA damage and toxicity. Furthermore, nuclear α-catenin is actively recruited to sites of DNA damage. This recruitment occurs in a β-catenin-dependent manner and requires nuclear actin polymerization. These findings provide mechanistic insight into the WNT-mediated regulation of the DNA damage response and suggest a novel role for the α-catenin–β-catenin complex in the nucleus.

KEY WORDS: α-Catenin, β-Catenin, DNA damage, Nuclear actin, WNT

INTRODUCTION

DNA damage leads to impaired cell function and maintenance (Jackson and Bartek, 2009). DNA breaks prompt a cascade of protein signaling events to identify lesions and efficiently repair these breaks to prevent mutagenesis. The accumulation of DNA damage, improper repair of DNA breaks and failure to remove cells with damaged DNA have been shown to contribute to oncogenesis (Bartek et al., 2007; Bartkova et al., 2005; Gorgoulis et al., 2005). This is especially true in adult stem cells, which accumulate cancer-driving mutations with age (Bartek et al., 2007; Liu et al., 2014; Sperka et al., 2012; Welch et al., 2012; Xie et al., 2014). Thus, insights into the sensitivity of cells to DNA damage have direct implications in understanding the molecular basis of cellular transformation as well as DNA damage-targeted chemotherapy.

The WNT pathway is a critical regulator of self-renewal, differentiation and the maintenance of cell stemness (Clevers and Nusse, 2012; Komiya and Habas, 2008). Aberrant activation of the WNT pathway has been closely linked to cellular transformation and aging (Barker et al., 2009; Polakis, 2000; Valenta et al., 2012). While WNT stimulation can trigger a number of protein cascades, the primary effector of the canonical WNT pathway is β-catenin (Valenta et al., 2012). WNT stimulation promotes β-catenin translocation into the nucleus, where β-catenin interacts with transcription factors and chromatin remodelers (Valenta et al., 2012). Another population of β-catenin is found at cell–cell adherens junctions; here, β-catenin binds cadherin proteins along with the F-actin-binding protein α-catenin, bridging the cytoskeleton and cellular junctions (McCrea et al., 2015; Valenta et al., 2012). This junctional population of β-catenin may also be responsive to WNT signaling (Hendriksen et al., 2008), suggesting that crosstalk between WNT signaling and adhesion complexes can be mediated by β-catenin (McCrea et al., 2015). Inactivating mutations in α-catenin, activating mutations in β-catenin and changes in the expression levels of these proteins have been repeatedly tied to various cancers (Aaltomaa et al., 1999; Anttila et al., 1998; Gofuku et al., 1999; Lifschitz-Mercer et al., 2001; Matsui et al., 1994; Nakopoulou et al., 2002; Richmond et al., 1997; Rimm et al., 1995; Shiozaki et al., 1994; Silvis et al., 2011; Tanaka et al., 2003; van Oort et al., 2007).

We and others have previously shown that αE-catenin (also known as CTNNA1; originally identified in epithelia, but now recognized as the most ubiquitously expressed α-catenin isoform, and thus hereafter referred to as α-catenin) can accumulate in the nucleus in a WNT/β-catenin-dependent manner (Choi et al., 2013; Daugherty et al., 2014; Giannini et al., 2000; Merdek et al., 2004). Nuclear α-catenin attenuates transcription of WNT pathway-responsive genes via β-catenin. α-Catenin can also influence general transcription by promoting nuclear actin polymerization, suggesting that α-catenin may antagonize transcription at β-catenin-regulated promoters by altering the local organization of nuclear actin (Daugherty et al., 2014; Serebryannyy et al., 2016b).

Here, we suggest that nuclear α-catenin influences the sensitivity of cells to DNA damage. We found that knockdown of α-catenin in a cell line that has abundant nuclear-localized α-catenin (Daugherty et al., 2014) resulted in increased numbers of DNA breaks and levels of histone 2 variant X phosphorylation (γH2AX), a marker of DNA damage, in response to treatment with etoposide, a topoisomerase II inhibitor and potent inducer of DNA breaks (Long et al., 1985; Schonn et al., 2010). By contrast, knockdown of β-catenin attenuated the levels of γH2AX induced by etoposide both in wild-type and α-catenin-knockdown cells. Colocalization and microirradiation experiments indicated that a nuclear population of α-catenin is actively recruited to sites of DNA damage, and this recruitment required binding to β-catenin as well as polymerized nuclear actin. Furthermore, WNT pathway stimulation or α-catenin overexpression reduced γH2AX levels, implying that there is a direct role for WNT signaling in the response to DNA damage.

RESULTS

Loss of α-catenin sensitizes cells to DNA damage

SW480 and DLD1 colorectal adenocarcinoma cells have truncations in adenomatous polyposis coli protein (APC), mimicking WNT activation, and resulting in the translocation of the α-catenin–β-catenin complex into the nucleus (Daugherty et al., 2014; Yang et al., 2006). To assess whether α-catenin mediates DNA damage sensitivity, we performed COMET assays on SW480 wild-type and α-catenin-knockdown cells treated with etoposide, and allowed DNA damage to resolve in fresh medium without

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etoposide (Fig. 1A). We found the incidence of DNA lesions to be higher both basally and upon etoposide treatment in α-catenin-knockdown cells, suggesting that α-catenin can influence the sensitivity of cells to DNA damage. Intriguingly, the clearance of DNA lesions was similar in both wild-type and in α-catenin-knockdown cells. Western blots showed that levels of the widely

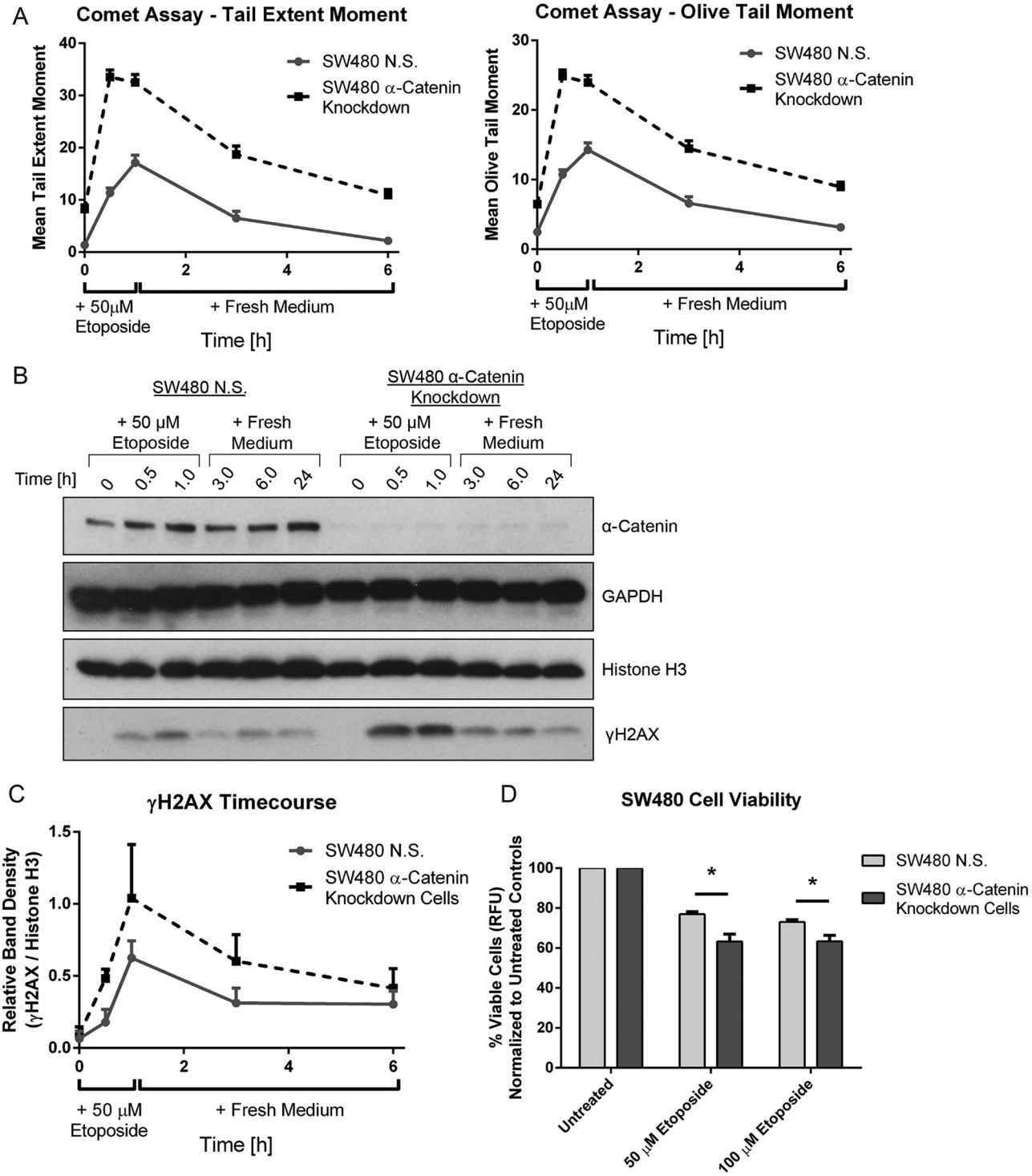

Fig. 1. α-Catenin suppresses DNA damage induction and associated toxicity in colon cancer cells. (A) SW480 cells stably transfected with non-specific (N.S.) and α-catenin shRNAs were left untreated or treated for 1 h with 50 μM etoposide, left to recover in fresh medium for 6 h and analyzed by COMET assay. COMET assays were performed after treatment with etoposide for 0.5 h and 1 h, and after fresh medium was added for 3 h and 6 h. Tail extent moment and olive tail moment were calculated as measures of DNA breaks. Data points show mean±s.e.m. ($n \geq 78$ cells in each group). (B) SW480 cells were prepared as in A. Following treatment as indicated, cells were lysed and blotted for γH2AX. (C) γH2AX band density relative to histone H3 was quantified. Mean+s.e.m. is shown ($n=4$). (D) SW480 cells stably transfected with non-specific (N.S.) and α-catenin shRNAs were treated for 48 h with the indicated concentrations of etoposide. The percentage of cells that remained viable was calculated relative to untreated controls. Mean+s.e.m. is shown ($n=3$). *$P<0.05$ (two-way ANOVA). Note the increased toxicity in SW480 α-catenin-knockdown cells as compared to that seen in wild-type cells.

Journal of Cell Science (2017) 130, 1717-1729 doi:10.1242/jcs.199893

used DNA damage marker γH2AX (Kuo and Yang, 2008) were higher in α-catenin-knockdown cells and remained elevated after DNA damage was allowed to resolve (Fig. 1B,C). The increase in DNA lesions and γH2AX levels correlated with decreased viability after etoposide treatment for 48 h (Fig. 1D), as previously described (Abe et al., 2008). Accordingly, we found that α-catenin-knockdown cells were less viable than wild-type cells.

Correspondingly, DLD1 α-catenin-knockdown cells, like SW480 cells, exhibited increased γH2AX levels after treatment for 1 h with etoposide and these levels remained elevated after a 6-h recovery period in medium without etoposide when compared to levels in wild-type cells (Fig. S1A,B). Prolonged treatment with etoposide for 48 h resulted in increased cell death in DLD1 α-catenin-knockdown cells as compared to wild-type cells (Fig. S1C). Although the differences in viability were modest, they confirmed that α-catenin-knockdown cells were more sensitive to etoposide.

β-Catenin mediates the sensitivity of cells to DNA damage

Because α-catenin is able to bind β-catenin in the nucleus and this interaction may be necessary for proper nuclear retention and function (Daugherty et al., 2014; McCrea and Gottardi, 2016), we next ascertained the importance of β-catenin in mediating the sensitivity of cells to DNA damage. β-Catenin knockdown in SW480 cells resulted in fewer DNA lesions following treatment with etoposide as measured using COMET assays (Fig. 2A). Furthermore, we noted a trend for γH2AX levels to be lower in β-catenin-knockdown cells as well as in β- and α-catenin co-knockdown cells after etoposide treatment (Fig. 2B,C). This result suggested that β-catenin may be necessary for the observed effect of α-catenin (Fig. 1B). To determine whether increased β-catenin signaling sensitizes cells to DNA damage, we expressed constitutively active β-catenin (S33Y) in U2OS cell. These cells, unlike SW480 cells, do not rely on aberrant WNT signaling for cell growth (Hadjihannas et al., 2012). β-Catenin (S33Y) expression resulted in an increase in the number of DNA lesions compared to the number in mock-transfected cells (Fig. 2D), as previously shown in thymocytes (Xu et al., 2008). Co-expression of constitutively active β-catenin with a Myc-tagged α-catenin partially alleviated the increase in the number of lesions (Fig. 2D). This is consistent with our finding that SW480 α-catenin-knockdown cells, which contain activated β-catenin, exhibited an increase in the number of DNA lesions (Fig. 1A).

α-Catenin is actively recruited to DNA damage repair foci

Despite the similar DNA lesion repair dynamics (Fig. 1A), the increased sensitivity to etoposide and elevated levels of γH2AX in SW480 α-catenin-knockdown cells suggested α-catenin might play a role in recognizing DNA damage and/or downstream DNA damage signaling (Fig. 1B; Fig. S1B). Therefore, we used WNT signaling-responsive MDCK cells to induce nuclear translocation of α-catenin by pre-treatment with lithium chloride (LiCl). LiCl treatment leads to the inactivation of GSK3 and simulates WNT pathway activation (Klein and Melton, 1996; Maher et al., 2009). LiCl-stimulated MDCK cells were either left untreated or treated with etoposide to induce DNA lesions, and incubated in medium without etoposide so that DNA damage repair foci could form. Cells were fixed and stained for endogenous α-catenin and for γH2AX to identify repair foci. LiCl-treated MDCK cells showed diffuse nuclear α-catenin staining, potentially localizing with sites of transcription (Fig. 3A; Daugherty et al., 2014). After treatment with LiCl and etoposide, α-catenin localized to DNA repair foci as marked by γH2AX, suggesting that nuclear α-catenin is recruited to sites of DNA damage (Fig. 3A). We also found instances where β-catenin, like α-catenin, localized to DNA damage repair foci in MDCK cells treated with LiCl and etoposide (Fig. 3B). To determine whether α-catenin is actively recruited to sites of DNA damage, we performed microirradiation experiments. U2OS cells were transfected with mCherry alone or with mCherry fused to a nuclear localization sequence (NLS)-tagged α-catenin, pre-treated with 30 mM LiCl and 1 μm regions were irradiated to monitor the recruitment of α-catenin to DNA lesions in real time (Fig. 3C). We noted a substantial and rapid enrichment of α-catenin at sites of DNA breaks after laser irradiation, suggesting that α-catenin can be specifically and actively recruited to sites of DNA damage.

α-Catenin requires β-catenin binding to mediate the DNA damage response

To better understand the mechanism by which α-catenin–β-catenin is involved in the DNA damage response, we assessed whether the interaction with β-catenin is necessary for α-catenin recruitment to repair foci. U2OS cells were transfected with full-length Myc–NLS-α-catenin (Fig. 4A), Myc–NLS-α-catenin without its β-catenin-binding domain (Myc–NLS-Δβ α-catenin; amino acids 82–906; Fig. 4B) or with just the actin-binding domain (ABD) of α-catenin (amino acids 697–906) conjugated to Myc and a NLS (Fig. 4C). U2OS cells were then pre-treated with 30 mM LiCl and DNA damage repair foci were formed through treatment with etoposide and recovered as above (Fig. 3A,B). Immunostaining for γH2AX to mark DNA repair foci and Myc to visualize α-catenin revealed that the β-catenin-binding domain is required for the proper localization of α-catenin to repair foci, in line with our expression studies (Fig. 2). To determine whether nuclear levels of α-catenin correlated with levels of γH2AX, we engineered SW480 cells stably knocked down for α-catenin to re-express Myc–NLS-α-catenin or Myc–NLS-Δβ-α-catenin. Expression of the full-length α-catenin construct was able to restore γH2AX levels to near those in wild type. However, this rescue effect was lost in the Myc–NLS-Δβ-α-catenin-expressing cells (Fig. S2A).

We next investigated how α-catenin–β-catenin could mediate the response to DNA damage. We performed pulldowns by using GST–α-catenin on nuclear extracts prepared from SW480 α-catenin-knockdown cells. Mass spectrometry to identify potential binding partners revealed a number of DNA damage proteins in our pulldown (Fig. S2B). Notable among these were poly(ADP-ribose) polymerase 1 (PARP1) and proteins of the Ku complex, which have been previously reported to bind β-catenin (Idogawa et al., 2007, 2005). To validate the proteomic results, we performed GST pulldowns with fragments of α-catenin, and blotted for PARP1. While the N-terminal, β-catenin-binding region and the full-length α-catenin protein were able to bind PARP1, the C-terminus of α-catenin and GST alone did not bind (Fig. S2C), indicating that binding of PARP1 to α-catenin may be mediated by β-catenin.

To assess whether PARP1 activity was necessary for α-catenin recruitment to sites of DNA damage, we performed microirradiation experiments in LiCl pre-treated U2OS cells transfected with mCherry–NLS-α-catenin in the presence or absence of the PARP inhibitor PJ34 (Fig. S2D). Incubation of U2OS cells with PJ34 before microirradiation abolished α-catenin recruitment to DNA lesions. Taken together, these data suggest that nuclear α-catenin requires β-catenin to be actively recruited to sites of DNA lesions along with DNA damage repair proteins such as PARP1.

Journal of Cell Science (2017) 130, 1717-1729 doi:10.1242/jcs.199893

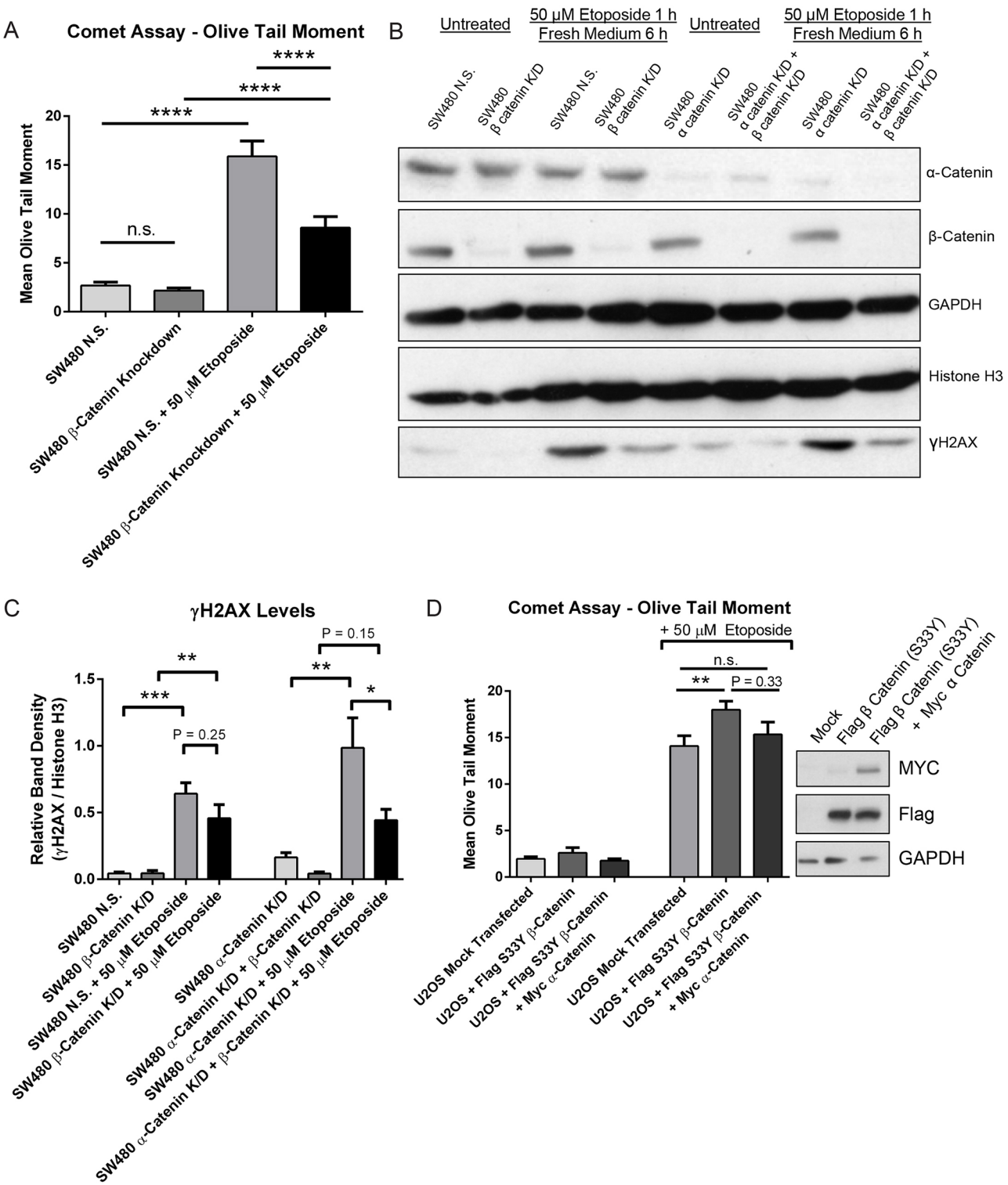

Fig. 2. β-Catenin suppresses DNA damage induction. (A) SW480 cells were transfected with non-specific siRNA (N.S.) or β-catenin siRNA pools for 72 h. Cells were then treated with 50 μM etoposide for 1 h and analyzed by performing COMET assays. Olive tail moment was calculated as a measure of DNA breaks. Mean+s.e.m. is shown ($n \geq 95$ cells in each group). ****$P<0.0001$; n.s., not significant (one-way ANOVA). Note the reduction in DNA damage in β-catenin-knockdown cells. (B) SW480 cells stably transfected with non-specific (N.S.) and α-catenin shRNAs were additionally transfected with non-specific siRNA (N.S.) or β-catenin siRNA pools for 72 h. Cells were then left untreated or treated with 50 μM etoposide for 1 h followed by recovery in fresh medium for 6 h, and analyzed by performing western blotting. Note the decreased levels of γH2AX in the β-catenin-knockdown cells irrespective of α-catenin expression. (C) Quantification of γH2AX band density normalized to histone H3 levels. Mean+s.e.m. is shown ($n=4$). *$P<0.05$; **$P<0.01$; ***$P<0.001$; n.s. not significant (one-way ANOVA). SW480 and SW480 α-catenin-knockdown cells were compared separately. (D) U2OS cells were mock transfected or transfected for 48 h with constitutively active β-catenin (S33Y) with or without wild-type Myc–α-catenin. Cells were pre-treated with 30 mM LiCl for 3 h, then left untreated or treated for 1 h with 50 μM etoposide and analyzed by performing a COMET assay. Olive tail moment was calculated as a measure of DNA breaks. Mean+s.e.m. is shown ($n \geq 76$ cells in each group). **$P<0.01$; n.s., not significant (one-way ANOVA). Western blots of the transfected proteins are also shown (right).

Journal of Cell Science (2017) 130, 1717-1729 doi:10.1242/jcs.199893

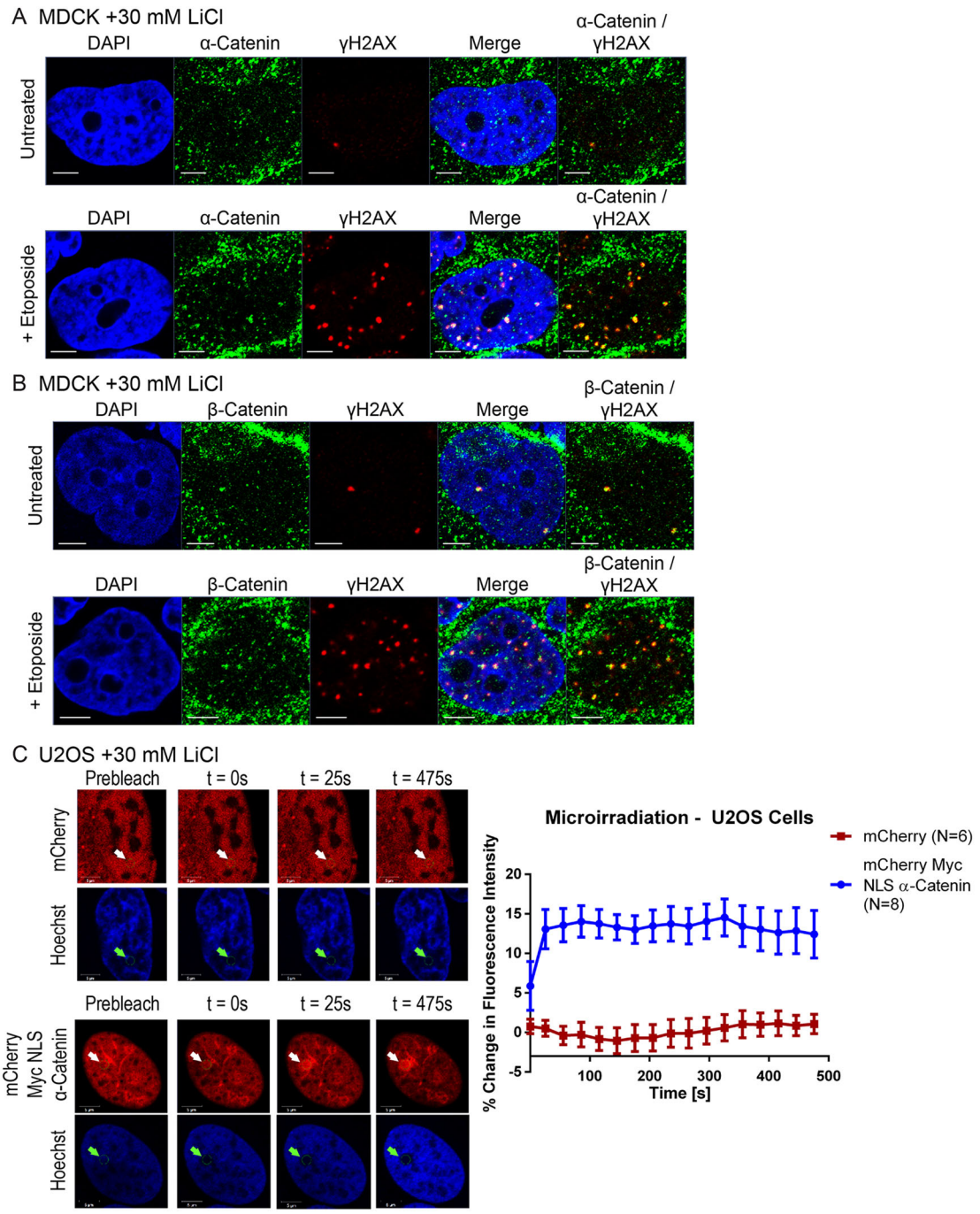

Fig. 3. α-Catenin is recruited to sites of DNA damage. (A) MDCK cells were pre-treated with 30 mM LiCl for 3 h (top) or pre-treated with 30 mM LiCl for 3 h followed by 50 µM etoposide for 1 h and incubated in fresh medium for 4 h to allow repair foci to form (bottom). MDCK cells were stained for endogenous α-catenin (green), γH2AX (red) and DAPI (blue). (B) MDCK cells were treated as in A and stained for endogenous β-catenin (green), γH2AX (red) and DAPI (blue). Note the localization of α- and β-catenin in DNA repair foci marked by γH2AX. Scale bars: 5 µm. (C) U2OS cells were transfected with mCherry (top) or mCherry fused to Myc–NLS–α-catenin (bottom) for 48 h. Cells were then incubated with 30 mM LiCl and Hoechst 33342 (5 µg/ml), to sensitize the cells, and microirradiated to induce localized sites of DNA damage. Cells were live-cell imaged to track the recruitment of mCherry (n=6) and mCherry–Myc–NLS-α-catenin (n=8) to sites of DNA damage. Green and white arrows denote the irradiation site (left). The relative fluorescence enrichment at sites of irradiation is shown as mean±s.e.m. (right).

Recruitment of α-catenin to sites of DNA damage requires nuclear actin

While the above data suggested that β-catenin binding is necessary for α-catenin-dependent modulation of the DNA damage response, it was not clear whether this domain was sufficient. Therefore, we transfected SW480 α-catenin-knockdown cells with mCherry, as a control, mCherry–NLS-α-catenin or an N-terminal fragment of α-catenin that contained the β-catenin-binding and homodimerization domains (amino acids 1–314) conjugated to a NLS and mCherry. Upon microirradiation, the N-terminal α-catenin construct exhibited impaired recruitment to irradiation sites as compared to full-length α-catenin (Fig. 5A). This suggests that the N-terminal, β-catenin-binding domain is not sufficient for recruitment of α-catenin to sites of DNA damage and that the

Journal of Cell Science (2017) 130, 1717-1729 doi:10.1242/jcs.199893

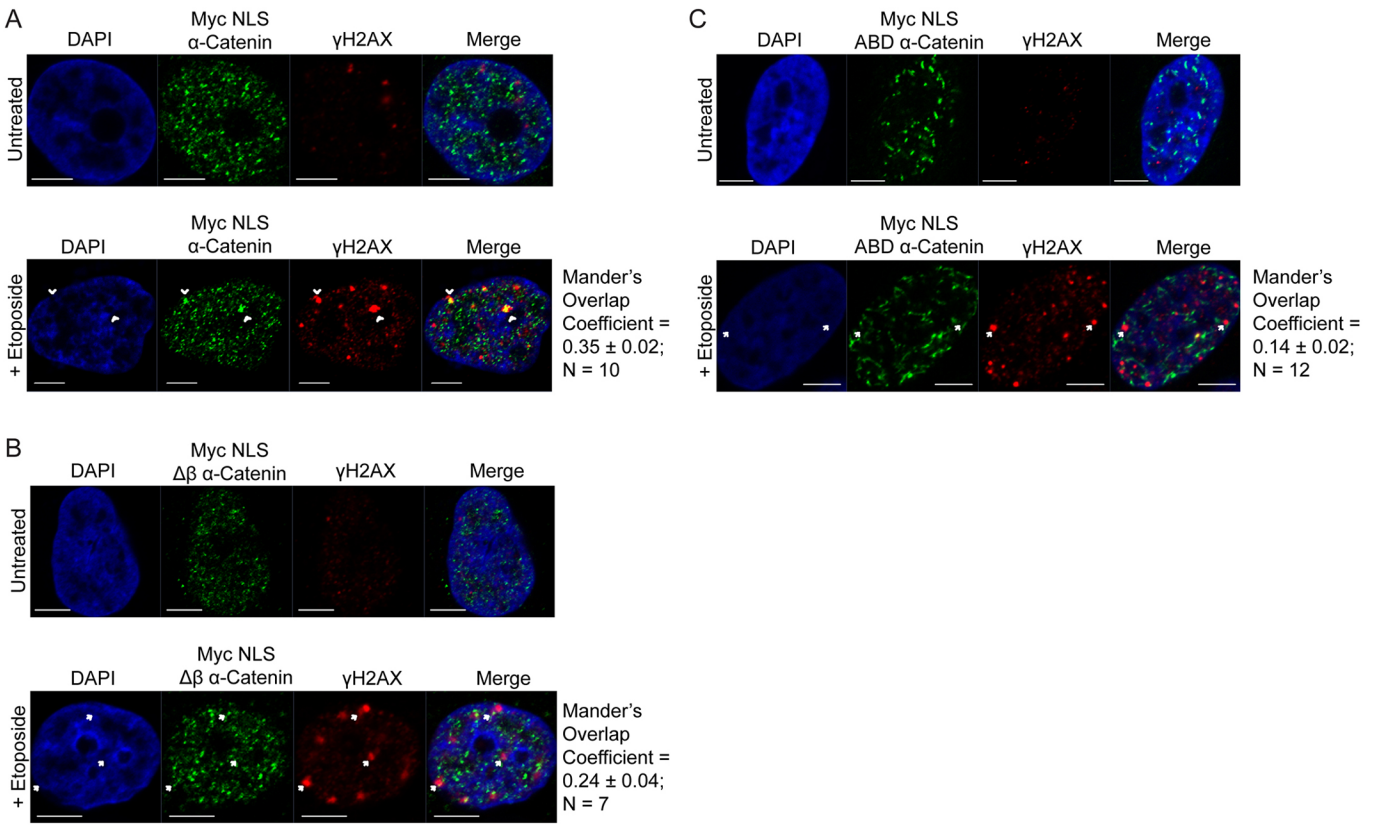

Fig. 4. The β-catenin-binding domain of α-catenin is necessary for recruitment to sites of DNA damage repair. U2OS cells transfected with (A) full-length Myc–NLS-α-catenin, (B) Myc–NLS-α-catenin without its β-catenin-binding domain (Myc–NLS-Δβ-α-catenin; amino acids 82–906), or (C) Myc–NLS tagged to just the actin-binding domain (ABD) of α-catenin (amino acids 697–906) for 48 h were pre-treated with 30 mM LiCl and left untreated (top) or treated with 50 µM etoposide for 1 h and left to recover for 4 h (bottom). Cells were stained for Myc to label α-catenin (green), γH2AX (red) to mark sites of DNA repair and DAPI (blue). Note the colocalization with the full-length but not the truncated α-catenin constructs (arrows). Manders' coefficients for Myc and γH2AX were obtained from images as shown (mean±s.e.m.). All values from treated cells showed statistically significant differences (one-way ANOVA). Scale bars: 5 µm.

C-terminus of α-catenin, which contains the F-actin-binding domain, is important for proper recruitment.

To test whether F-actin binding was a requisite for α-catenin recruitment to DNA lesions, SW480 α-catenin-knockdown cells were co-transfected with mCherry–NLS-α-catenin and a series of EYFP–β-actin constructs with different polymerization properties. EYFP–NLS-S14C-β-actin localizes to the nucleus and stabilizes actin polymerization, while EYFP–NLS-G13R and -R62D-β-actin localize to the nucleus and resist polymerization (Posern et al., 2002; Serebryannyy et al., 2016b). Wild-type EYFP–β-actin was used as a control. Microirradiation experiments were then performed and recruitment of α-catenin was assessed (Fig. 5B,C). We found NLS-S14C-β-actin promoted α-catenin recruitment, while NLS-R62D and -G13R-β-actin both attenuated α-catenin recruitment. However, we did not detect EYFP–β-actin nor EYFP–NLS-S14C β-actin recruitment to DNA lesions (Fig. S3A). The F-actin probe Lifeact-NLS-RFP (Riedl et al., 2008) similarly showed no signs of recruitment (Fig. S3B). These results suggest that α-catenin is able to bind polymeric nuclear actin at sites of DNA breaks, and this interaction appears to be necessary for proper α-catenin recruitment.

WNT stimulation mediates the DNA damage response

Given the data implicating α- and β-catenin in the DNA damage response, we investigated how WNT signaling influences the sensitivity to DNA damage. As previously reported (Daugherty et al., 2014; Merdek et al., 2004), WNT pathway activation increased both α- and β-catenin translocation into the nucleus (Fig. 6A). Furthermore, pre-treating cells with LiCl decreased γH2AX levels induced by etoposide as compared to control NaCl treatment (Fig. 6B), suggesting that α-catenin bound to β-catenin in the presence of WNT pathway activation can decrease the sensitivity to DNA damage. To examine whether α-catenin may be responsible for the reduced levels of DNA damage, MDCK cells stably overexpressing EGFP–α-catenin were pre-treated with LiCl, then treated with etoposide, and DNA damage was assessed by monitoring the levels of γH2AX (Fig. 6C). Indeed, EGFP–α-catenin-overexpressing cells exhibited lower γH2AX levels. To confirm that α-catenin-knockdown cells were more chemosensitive, we pre-treated MDCK cells, MDCK α-catenin-knockdown cells and MDCK α-catenin-knockdown cells expressing EGFP–α-catenin with LiCl. Cells were then treated with etoposide for 48 h and viability was measured (Fig. 6D). We found α-catenin-knockdown cells were more susceptible to cell death induced by etoposide treatment and that re-expression of α-catenin rescued cell viability.

To assess whether receptor activation of the WNT pathway recapitulated the results seen with LiCl treatment, MDCK cells were infected with WNT3a–GFP adenovirus or GFP adenovirus, as a control, to stimulate the WNT pathway (Lam et al., 2011). Infection with WNT3a adenovirus increased translocation of endogenous α-catenin into the nucleus (Fig. 7A). Infected MDCK cells were then treated with etoposide to induce DNA lesions, and γH2AX levels were assessed (Fig. 7B). WNT3a–GFP-infected MDCK cells stably knocked down for α-catenin exhibited increased γH2AX levels after

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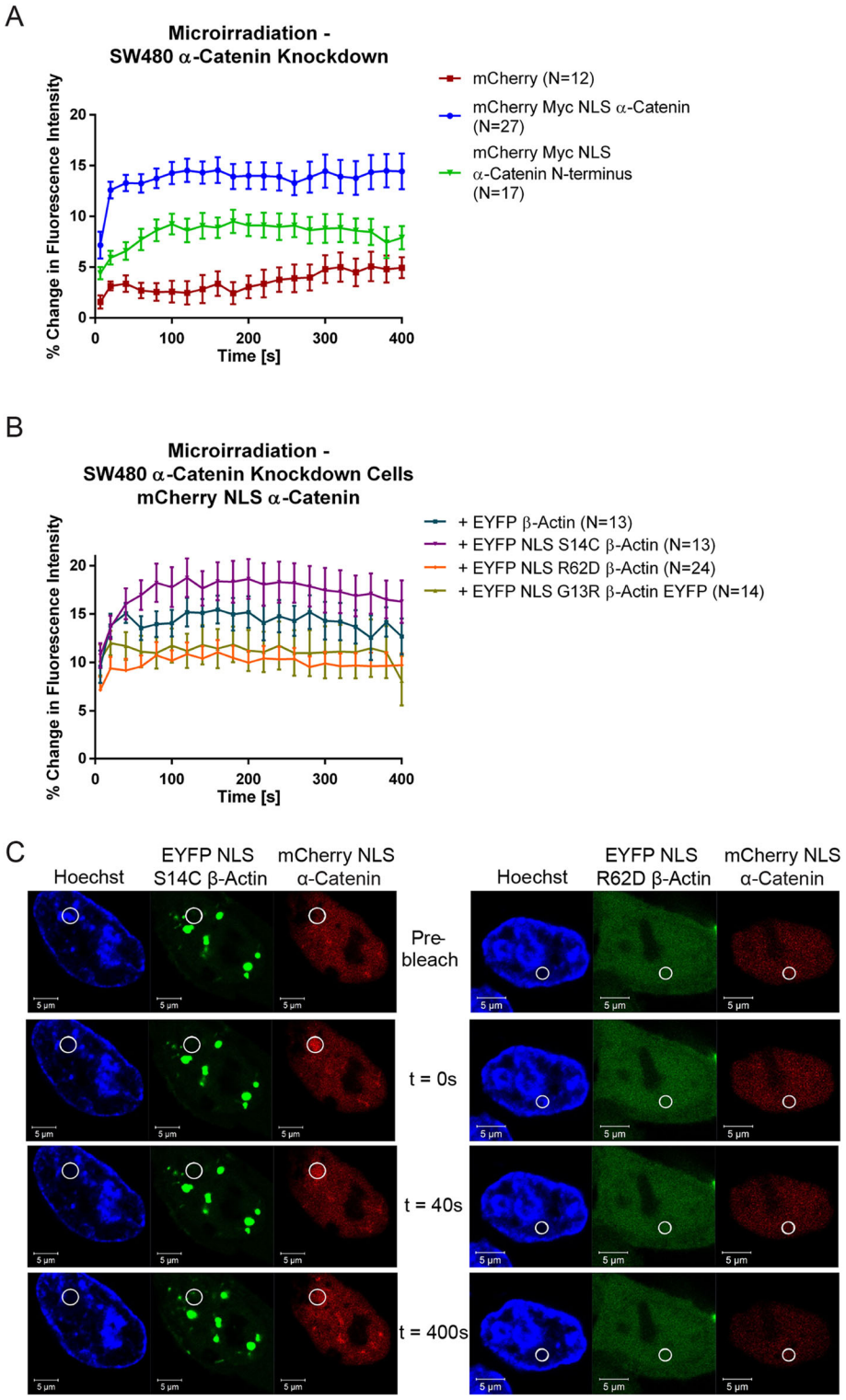

Fig. 5. Polymerized nuclear actin enhances α-catenin localization at sites of DNA damage. (A) SW480 α-catenin-knockdown cells were transfected for 48 h with mCherry (*n*=12), mCherry–Myc–NLS-α-catenin (*n*=27) or mCherry–Myc–NLS-α-catenin containing only the N-terminal β-catenin-binding and homodimerization domains (amino acids 1–314; *n*=17). Cells were then incubated with Hoechst 33342 (5 µg/ml), to sensitize cells, and microirradiated to induce localized sites of DNA damage. Note the reduced response when only the N-terminus of α-catenin is expressed. (B) SW480 α-catenin-knockdown cells were co-transfected with mCherry–Myc–NLS-α-catenin and EYFP–β-actin (*n*=13), as a control, EYFP–NLS-β-actin containing actin depolymerization-promoting mutations (EYFP–NLS-R62D-β-actin, *n*=24; EYFP–NLS-G13R-β-actin, *n*=14), or a polymerization-promoting mutation (EYFP–NLS-S14C-β-actin, *n*=13) for 48 h. Cells were then incubated with 30 mM LiCl and Hoechst 33342 (5 µg/ml), to sensitize the cells, and microirradiated to induce localized sites of DNA damage. Cells were live-cell imaged to track the movement of mCherry–Myc–NLS-α-catenin to the sites of damage. Quantification of fluorescence enrichment is shown as mean±s.e.m. Actin depolymerization attenuated α-catenin recruitment to sites of DNA damage, while actin polymerization enhanced α-catenin recruitment. (C) Representative experiments showing mCherry–Myc-NLS-α-catenin recruitment in cells co-transfected with EYFP–NLS-S14C-β-actin (left panels) or EYFP–NLS-R62D-β-actin (right panels). White circles denote the sites of irradiation.

etoposide treatment, and these levels could be decreased by stably re-expressing EGFP–α-catenin (Fig. 7C). To assess cell viability, MDCK cells, MDCK α-catenin-knockdown cells and MDCK α-catenin-knockdown cells expressing EGFP–α-catenin were infected with WNT3a–GFP adenovirus and treated with etoposide for 48 h (Fig. 7D). We found that MDCK α-catenin-knockdown cells appeared to be more susceptible to cell death, and this effect could be partially rescued by re-expression of EGFP–α-catenin. Taken together, these data suggest that WNT pathway activation can decrease the susceptibility of cells to DNA damage and that this effect is mediated by α-catenin.

DISCUSSION

While α-catenin is largely regarded as a junctional protein, a substantial amount of α-catenin is found in the cytoplasm, where it regulates the cytoskeleton (Benjamin et al., 2010; Bianchini et al., 2015), and the nucleus, where it has been implicated in transcription (Choi et al., 2013; Daugherty et al., 2014; Giannini et al., 2000;

Journal of Cell Science (2017) 130, 1717-1729 doi:10.1242/jcs.199893

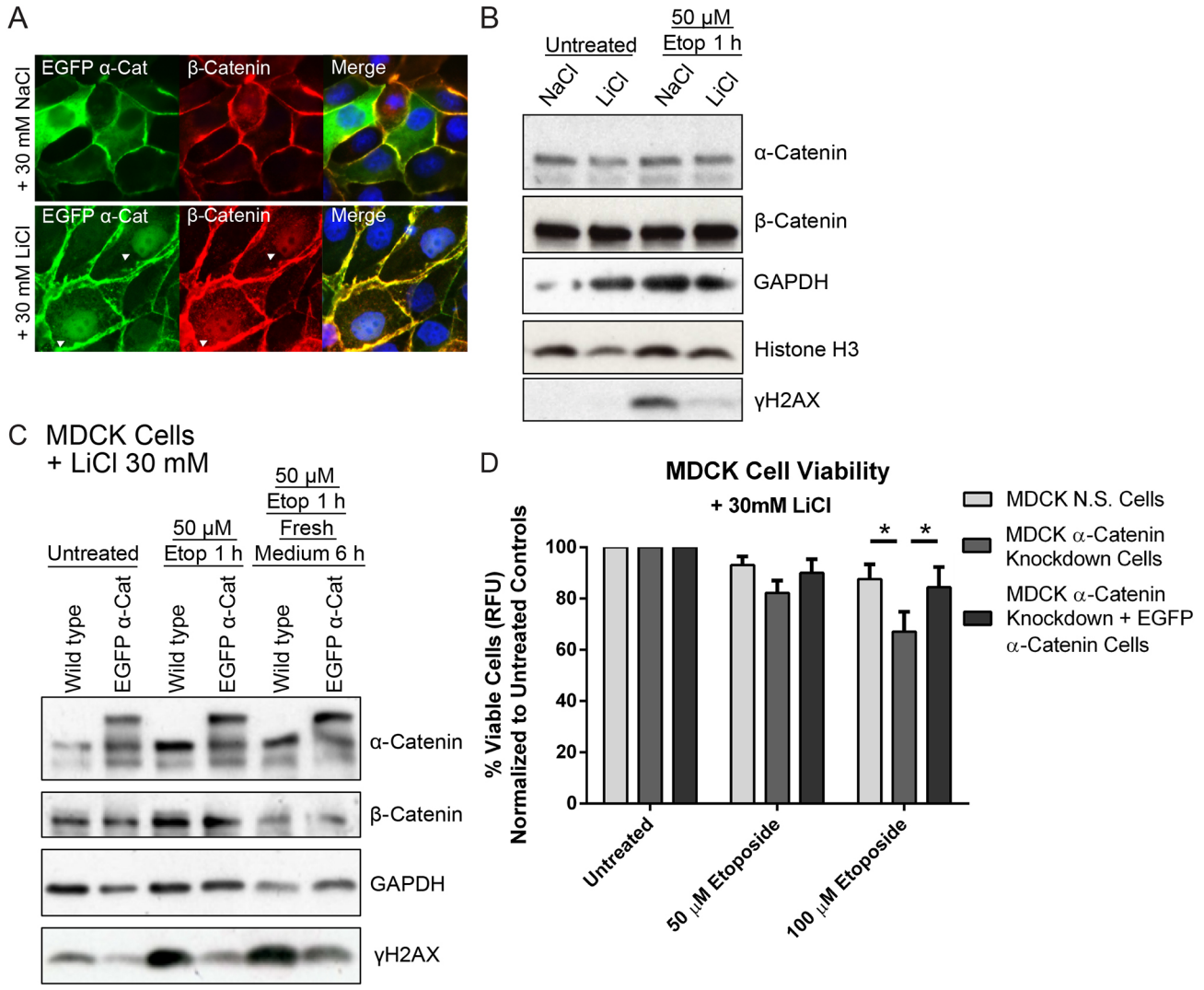

Fig. 6. α-Catenin suppresses DNA damage induction and toxicity with WNT pathway activation. (A) MDCK cells expressing EGFP–α-catenin (EGFP α-Cat; green) were treated with 30 mM NaCl as a control (top) or 30 mM LiCl to inhibit GSK3 and increase WNT signaling (bottom). Cells fixed and stained for β-catenin (red) and DAPI (blue) showed increased levels of EGFP–α-catenin in the nucleus (arrowheads). (B) MDCK cells were treated with 30 mM NaCl or 30 mM LiCl for 3 h then treated with 50 μM etoposide for 1 h. Whole-cell extracts were prepared and immunoblotted. Decreased γH2AX levels were observed in cells stimulated with LiCl. (C) Wild-type MDCK cells or MDCK cells overexpressing EGFP–α-catenin were pre-treated with 30 mM LiCl for 3 h then treated with 50 μM etoposide for 1 h, treated with 50 μM etoposide for 1 h then incubated in fresh medium for 6 h to recover, or left untreated. Whole-cell extracts were prepared and immunoblotted. Note the correlation between α-catenin expression and γH2AX levels. (D) Stable MDCK cells infected with a non-specific hairpin (N.S.), knocked down for α-catenin, and knocked down cells rescued with EGFP–α-catenin were concurrently treated with 30 mM LiCl and the indicated concentrations of etoposide for 48 h. The percentage of cells that remained viable was calculated relative to that in the untreated controls. α-Catenin-knockdown cells exhibited increased toxicity as compared to their wild-type counterparts and EGFP–α-catenin rescued cells (mean+s.e.m.; n=5). *P<0.05 (two-way ANOVA).

Merdek et al., 2004). Here, we show that nuclear α-catenin is actively recruited to sites of DNA damage (Fig. 3), and the levels of α-catenin–β-catenin correspond to the sensitivity of cells to DNA lesions (Figs 1, 6 and 7, Fig. S2).

The interaction of the nuclear α-catenin–β-catenin complex with DNA repair proteins upon WNT pathway activation may help maintain genomic stability as well as facilitate efficient DNA damage recognition and recruitment to sites of repair. In support of this idea, α-catenin proteomic analysis suggested that nuclear α-catenin is able to bind to several DNA recognition and repair proteins (Fig. S2), including PARP1 and Ku70 (also known as XRCC6), which have been reported to competitively interact with β-catenin to regulate transcription (Idogawa et al., 2007, 2005; Zhu et al., 2016). The effect of α-catenin knockdown on the DNA damage response (Fig. 1) and the necessity of PARP1 activity for proper α-catenin recruitment to sites of DNA damage (Fig. S2D) are in line with results from PARP1-knockout models. PARP1-knockout mice show increased sensitivity to DNA damage and genotoxic stress (de Murcia et al., 1997; Wang et al., 1997), and mouse embryonic fibroblasts derived from PARP1-deficient mice show impaired recruitment of other DNA damage repair proteins (Haince et al., 2008). Similarly, cells deficient for Ku70 and/or Ku80 (Ku80 is also known as XRCC5) are hypersensitive to etoposide treatment (Ayene et al., 2005; Jin et al., 1998). Notably, these DNA repair factors are involved in the initial recognition of DNA lesions, and α-catenin accumulates rapidly at sites of DNA damage (Fig. 3); hence, α-catenin may be involved early in the detection of DNA lesions and downstream signaling. Furthermore, given the role of α-catenin and β-catenin in transcription (Choi et al., 2013; Daugherty et al., 2014; Idogawa et al., 2007; Valenta et al.,

Journal of Cell Science (2017) 130, 1717-1729 doi:10.1242/jcs.199893

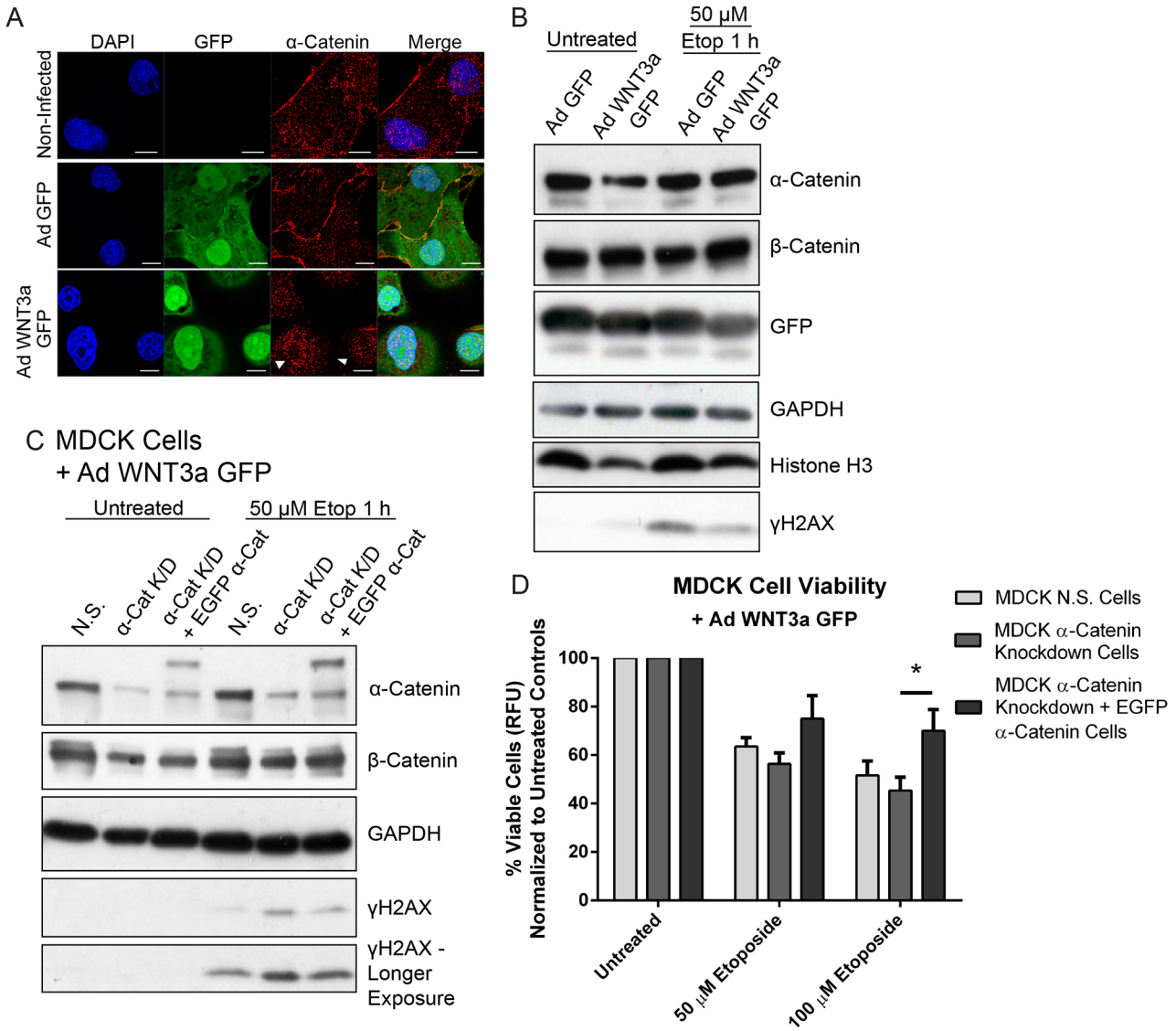

Fig. 7. α-Catenin suppresses DNA damage induction and toxicity in a WNT-dependent manner. (A) MDCK cells were left untreated (top), or infected with Ad GFP (middle) or Ad WNT3a–GFP (bottom) for 24 h. Cells were then fixed and stained for α-catenin. Ad WNT3a–GFP-infected cells exhibited increased nuclear localization of α-catenin (arrowheads). Scale bars: 10 μm. (B) MDCK cells were infected with Ad GFP or Ad WNT3a–GFP for 24 h then treated with 50 μM etoposide for 1 h. Immunoblots of whole-cell extracts show that there is a decreased level of γH2AX in cells stimulated with WNT3a–GFP. (C) Stable MDCK cells infected with a non-specific hairpin (N.S.) shRNA lentivirus, knocked down for α-catenin (α-Cat K/D), and knocked down cells rescued with EGFP–α-catenin (EGFP α-Cat) were infected with Ad WNT3a–GFP for 24 h followed by a 1-h treatment with 50 μM etoposide. Immunoblots of whole-cell extracts show that there is a correlation between increased α-catenin expression and decreased γH2AX levels. (D) Same as in Fig. 6D except cells were infected with WNT3a–GFP for 24 h then treated for 48 h with the indicated concentrations of etoposide (mean+s.e.m.; n=3). *P<0.05 (two-way ANOVA). Increased toxicity was observed in α-catenin-knockdown cells as compared to their wild-type counterparts and EGFP–α-catenin-expressing cells.

2012), the recruitment of these proteins to sites of DNA lesions may also regulate the transcriptional response to DNA damage in a WNT-dependent manner.

Our study builds on previous work implicating β-catenin in the regulation of genome stability (Aoki et al., 2007; Dose et al., 2014) and in the DNA damage response (Chandra et al., 2015; Priolli et al., 2013; Tavana et al., 2013; Xu et al., 2008; Zhang et al., 2011). Indeed, our data align well with these previous reports. They suggest that increased unregulated or oncogenic translocation of β-catenin into the nucleus sensitizes cells to DNA lesions (Fig. 2). We also provide evidence for a new level of WNT and β-catenin regulation of DNA damage via α-catenin. This is in agreement with numerous studies showing that gain-of-function mutations in β-catenin signaling and loss of α-catenin regulation are prevalent in cancer (Aaltomaa et al., 1999; Anttila et al., 1998; Clevers and Nusse, 2012; Gofuku et al., 1999; Lifschitz-Mercer et al., 2001; Matsui et al., 1994; Nakopoulou et al., 2002; Polakis, 2000; Richmond et al., 1997; Rimm et al., 1995; Shiozaki et al., 1994; Silvis et al., 2011; Tanaka et al., 2003; van Oort et al., 2007; Yang et al., 2006). This additional level of regulation by α-catenin may help explain why WNT stimulation has been reported to decrease the sensitivity of cells to DNA damage despite increased nuclear β-catenin levels (Chandra et al., 2015; Chen et al., 2007; Jun et al., 2016; Woodward et al., 2007), and why different experimental systems have had confounding results (Chevillard-Briet et al., 2014; Orford et al., 1999; Tao et al., 2015; Watson et al., 2010). Intriguingly, p53 is able to regulate WNT ligand production in a cell type-dependent manner (Lee et al., 2010) as well as β-catenin levels (Kim et al., 2011; Sadot

Journal of Cell Science (2017) 130, 1717-1729 doi:10.1242/jcs.199893

et al., 2001), suggesting a complicated interplay between the DNA damage response and WNT signaling.

Our results suggest that the effect of WNT stimulation on the DNA damage response may depend on the levels of nuclear α-catenin, as well as those of β-catenin and other proteins recruited to this complex. Intriguingly, loss of APC, a component of the β-catenin destruction complex and actin-nucleating factor (Moseley et al., 2007; Okada et al., 2010), has also been found to increase DNA damage and genomic instability (Aoki et al., 2007; Fodde et al., 2001a; Meniel et al., 2015). APC has been shown to translocate into the nucleus, bind both α- and β-catenin and inhibit transcription (Choi et al., 2013), which is reminiscent of the role of nuclear α-catenin (Daugherty et al., 2014; McCrea and Gottardi, 2016). Furthermore, nuclear APC is directly recruited to sites of DNA damage repair (Kouzmenko et al., 2008) and has been implicated in base excision repair (Narayan and Sharma, 2015). Notably, the majority of colon cancers have mutations in APC leading to protein truncation (Fodde et al., 2001b; Kinzler and Vogelstein, 1996; Smith et al., 1993), and deletion of APC in crypt stem cells in mice leads to transformation within days (Barker et al., 2009). α-Catulin (also known as CTNNAL1), a vinculin-related protein with homology to α-catenin, has also been shown to increase γH2AX levels when knocked down and may regulate cell fate (Fan et al., 2011). Taken together, these studies suggest the WNT pathway may be an important regulator of how the cell responds to DNA damage, and the mechanisms that regulate catenin activity in the cytoplasm may be conserved in the nucleus.

While β-catenin binding is necessary for proper recruitment of α-catenin (Fig. 4), we find that the C-terminus, which contains an F-actin-binding domain, is also important for localization of α-catenin to sites of DNA damage (Fig. 5). Previous studies have shown polymerized actin is necessary for retention of Ku80 at sites of DNA damage (Andrin et al., 2012), and DNA damage has been shown to increase nuclear actin polymerization, potentially inducing formation of nuclear actin filaments that may play a role in nuclear oxidation (Belin et al., 2015) as well as the recruitment and activity of β-catenin (Yamazaki et al., 2016). Although we noted no evidence of nuclear actin filament formation, our study supports a model whereby recruitment of nuclear α-catenin stabilizes polymerized actin at sites of DNA damage (Fig. 5). Polymerizing nuclear actin may act as a scaffold for other repair proteins at sites of DNA damage (Andrin et al., 2012), locally alter nuclear actin dynamics leading to downstream changes in transcription and chromatin remodeling (de Lanerolle and Serebryannyy, 2011; Serebryannyy et al., 2016a,b), or tether repair factories to the nuclear matrix and other nuclear subcompartments (Koehler and Hanawalt, 1996; Mahen et al., 2013; Marnef and Legube, 2017). Notably, lamins are known to regulate genomic stability as well as DNA damage repair (Gonzalo, 2014) and have been shown to interact with nuclear actin (Ho et al., 2013; Plessner et al., 2015; Simon et al., 2010). Multiple studies have identified other actin regulatory proteins that are able to translocate into the nucleus and are involved in the DNA response including JMY (Lin et al., 2014; Zuchero et al., 2009), filamin A (Yue et al., 2013), Arp5 (Kitayama et al., 2009), APC (Kouzmenko et al., 2008; Meniel et al., 2015; Narayan and Sharma, 2015), formin-2, spire-1/2 (Belin et al., 2015), myosin VI (Jung et al., 2006) and nuclear histone deacetylases (HDACs; Serebryannyy et al., 2016a), as well as p53, which is speculated to directly bind F-actin (Metcalfe et al., 1999). Furthermore, elegant *in vitro* studies have demonstrated that α-catenin forms a catch bond with F-actin when bound to β-catenin (Buckley et al., 2014). Thus, it is plausible that α-catenin acts as a force-dependent molecular tether for efficient maintenance of DNA lesions or chromatin state.

In summary, we provide evidence that α-catenin is able to regulate the WNT/β-catenin-mediated response to DNA damage. While we cannot fully exclude the influence of cytoplasmic α-catenin and further study is necessary to fully delineate the mechanism by which the α-catenin–β-catenin complex affects the sensitivity and response to DNA lesions, we find that the nuclear α-catenin–β-catenin complex at sites of DNA damage functionally parallels the α-catenin–β-catenin complex at adherens junctions. The interaction with β-catenin targets α-catenin to DNA lesions, whereas binding to nuclear actin may serve to tether the protein complex or recruit additional factors. Additionally, our data suggest that the correlation between mutations in the WNT pathway and oncogenesis may be tied to increased susceptibility to DNA mutagenesis as well as anchorage-independent growth.

MATERIALS AND METHODS

Cell lines, constructs, and reagents

SW480, MDCK and DLD1 cell lines were established and cultured as previously described (Daugherty et al., 2014; Escobar et al., 2015). U2OS cells were obtained from American Type Culture Collection. Cells were cultured in Dulbecco's modified Eagle's medium (Corning) supplemented with 1% penicillin-streptomycin (Invitrogen) and 10% fetal bovine serum (Invitrogen). Cells were incubated at 37°C and with 5% CO_2. 10 µg/ml puromycin (Santa Cruz) was used to maintain stable cell lines when necessary. Cells were regularly checked for contamination. Where indicated, cells were treated with etoposide (Enzo).

Transient DNA transfections were performed using Polyjet (SignaGen). siRNA transfections were carried out using Lipofectamine 3000 (Invitrogen) according to the manufacturer's protocol. For β-catenin knockdown, siGENOME SMART pool human CTNNB1 (M-003482-00; Thermo Scientific) was used and ON-TARGETplus Control Pool (D-001810-10-05; Thermo Scientific) siRNA was used as a non-specific control.

Adenoviral infection was carried out overnight with Ad WNT3a–GFP or Ad GFP, which were kind gifts from Dr Tong Chuan He (University of Chicago, Chicago, IL).

GST- and Myc-tagged α-catenin and truncation constructs were as previously described (Daugherty et al., 2014). To generate mCherry–Myc–NLS-α-catenin and the corresponding mCherry–Myc–NLS-tagged N-terminal fragment of α-catenin, Myc–NLS-α-catenin was digested using restriction enzymes EcorI/ApaI, purified, and inserted into the pmCherry-C1 vector backbone (Clonetech). The EYFP–NLS-β-actin constructs and mutations were previously described (Chang et al., 2011; Posern et al., 2002). Lifeact-NLS-RFP was created by PCR mutagenesis from Lifeact-RFP, a kind gift from Dr Alexander Bershadsky (Weizmann Institute of Science, Israel).

Primary antibodies used in this study were against α-catenin [antibody 5B11 (Daugherty et al., 2014) for immunostaining (1:50), and sc-7894 (Santa Cruz Biotechnology) for immunoblotting (1:5000)], β-catenin [2E1 for immunostaining (1:50) and dephosphorylated β-catenin (Santa Cruz Biotechnology) for immunoblotting (1:5000)], Myc (9E10, Santa Cruz; 1:200), GAPDH (6C5, Abcam; 1:10,000), GFP (ab290, Abcam; 1:10,000), histone H3 (A300-823A, Bethyl; 1:5000), HDAC1 (A300-713A, Bethyl; 1:5000), and γH2AX (A300-081A, Bethyl; 1:1000 for immunostaining; 1:10,000 for immunoblotting). Secondary antibodies used were goat IgGs conjugated to Dylight 488 (Thermo Scientific), Texas Red (Jackson Labs) or Cy3 (Jackson Labs) and were used at 1:200. Mounting medium containing DAPI (Vectashield) was used for immunocytochemistry. Primary antibody binding in western blots was detected with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Labs; 1:10,000) using ECL reagent (Denville).

Immunostaining and microscopy

Cells were plated on glass coverslips at least 24 h before fixation or transfection. Cells were fixed in 4% paraformaldehyde (PFA) for 10 min,

then permeabilized with 0.3% Triton X-100 (Sigma-Aldrich) in PBS for 7 min. After permeabilization, cells were washed with PBS and incubated in 2% bovine serum albumin (BSA) in PBS for 1 h at room temperature. Cells were stained in a humidity chamber. Primary antibody was added for 1 h at room temperature or overnight at 4°C. Cells were then washed with PBS and secondary antibody was added for 1 h at room temperature. Cells were washed a final time and mounted in Vectashield containing DAPI. Confocal images were obtained by using a Zeiss LSM 710 confocal microscope. Acquired images were analyzed using Zeiss Zen software. Manders' coefficients were calculated using the Foci Counter ImageJ plugin (University of Konstanz Bioimaging Center Toolkit).

Microirradiation

SW480 α-catenin-knockdown cells or U2OS cells were transfected with the indicated constructs for 48 h on glass-bottom dishes (Mattek). Before imaging, cells were washed and incubated in DMEM without Phenol Red. U2OS cells were pre-treated with 30 mM LiCl 1 h before imaging and with PJ34 (10 µM) or Mirin (100 µM) (Santa Cruz Biotechnology) for 1 h where indicated. SW480 α-catenin-knockdown cells and U2OS cells were incubated in 5 µg/ml Hoechst 33342 (Santa Cruz Biotechnology) 15 min before imaging to pre-sensitize cells to irradiation. Cells were imaged at 37°C on a Zeiss 710 confocal microscope with a 40×1.4 NA oil alpha Plan-Apochromat objective. Irradiation was performed using a 405 nm laser at 100% power in a ∼1 µm circle for 25 iterations. Images were collected every 20 s for 25 cycles.

Viability assays

Viability assays were performed with PrestoBlue reagent (Invitrogen) according to the manufacturer's protocol. Briefly, cells were plated in 96-well plates for 24 h before treatment with the indicated concentration of etoposide for 48 h. Cells were then incubated in PrestoBlue reagent (1:10; reagent:medium) and viability was assessed by determining the level of fluorescence with a microplate reader. Where indicated, cells were incubated with 30 mM LiCl or infected with Ad Wnt3a–GFP 24 h after plating, followed by etoposide 24 h after pre-treatment.

COMET assays

COMET assays were performed using the COMET SCGE assay kit (Enzo, Trevigen). Wild-type SW480 and knockdown cells were left untreated or were treated with etoposide for the indicated periods then combined (10^5 cells/ml) with molten LMAgarose (1:10; v/v). Transfected U2OS and MDCK cells were pre-treated with 30 mM LiCl for 3 h before etoposide addition. After combining with agarose, cells were placed on glass slides and immersed in lysis solution [2.5 M NaCl, 100 mM EDTA (pH 10), 10 mM Tris base, 1% sodium lauryl sarcosinate, 1% Triton X-100] for 1 h, then immersed in alkaline solution (NaOH 12 µg/ml and 1 mM EDTA) for 1 h. Slides were washed in 1× TBE (89 mM Tris, 89 mM boric acid and 2 mM EDTA), placed in an electrophoresis chamber in 1× TBE, and run at 18 V for 10 min. Slides were washed in 70% ethanol, dried and stained with CYGREEN dye (Enzo) to label DNA. Slides were imaged by using a BX51 fluorescence microscope (Olympus) and analyzed with the Image J plugin OpenComet.

GST pulldown assays

Plasmids for expression of GST-tagged α-catenin variants were made using the PGEX vector backbone and transformed in BL21 cells. To perform pulldowns, transformed bacterial cells were sonicated in binding buffer [20 mM HEPES pH 6.8, 150 mM KOAc, 250 mM sorbital, 2 mM Mg $(OAc)_2$, containing protease inhibitors and DNase I] for 10 min in 30 s intervals. Lysates were spun at 20,000 ***g*** for 10 m at 4°C to pellet cellular debris. Cleared lysates (5 mg) were then incubated with 50 µl of 50% immobilized glutathione–agarose beads (Thermo-Scientific) for 2 h at 4°C. Beads were washed repeatedly in binding buffer. Washed beads were incubated in SW480 α-catenin-knockdown nuclear extract (1 mg) and incubated overnight at 4°C. Following incubation, beads were again washed repeatedly in binding buffer to remove non-specific interactions. To elute bound protein, beads were incubated with 10 mM reduced glutathione (Sigma-Aldrich) in 50 mM Tris-HCl, pH 8.0 at room temperature with agitation. Proteomic experiments were performed by the CBC-UIC Research Resources Center Mass spectrometry, Metabolomics and Proteomics Facility. Analysis of proteomics data was performed using Scaffold proteome software.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: L.A.S., C.J.G., P.d.L.; Methodology: L.A.S., A.Y., C.J.G., P.d.L.; Formal analysis: L.A.S.; Investigation: L.A.S.; Resources: A.Y., C.J.G., P.d.L.; Writing - original draft: L.A.S.; Writing - review & editing: L.A.S., C.J.G., P.d.L.; Visualization: L.A.S.; Supervision: L.A.S., C.J.G., P.d.L.; Project administration: L.A.S., C.J.G., P.d.L.; Funding acquisition: L.A.S., C.J.G., P.d.L.

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Supplementary information

Supplementary information available online at
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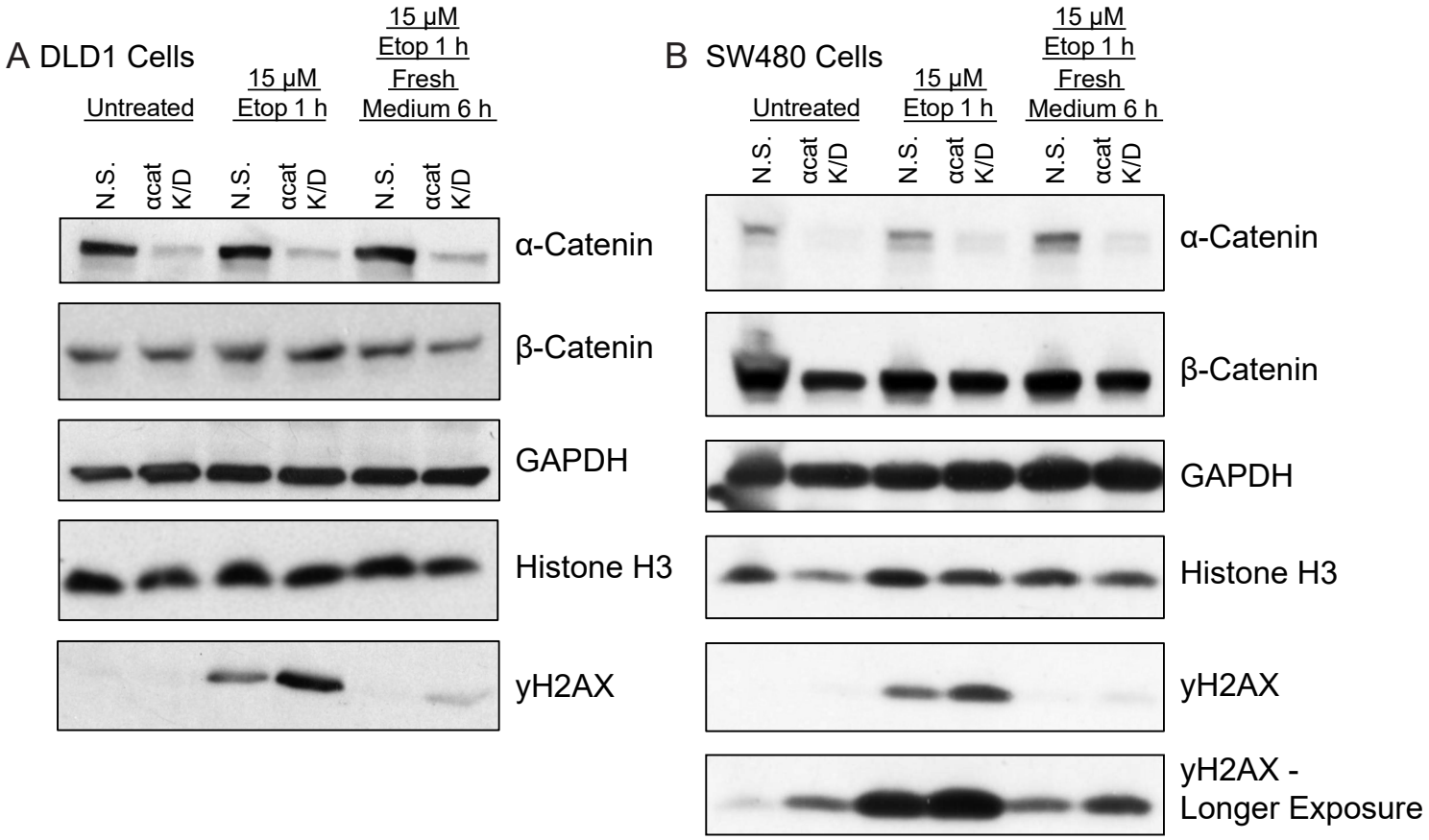
SUPPLEMENTARY FIGURES



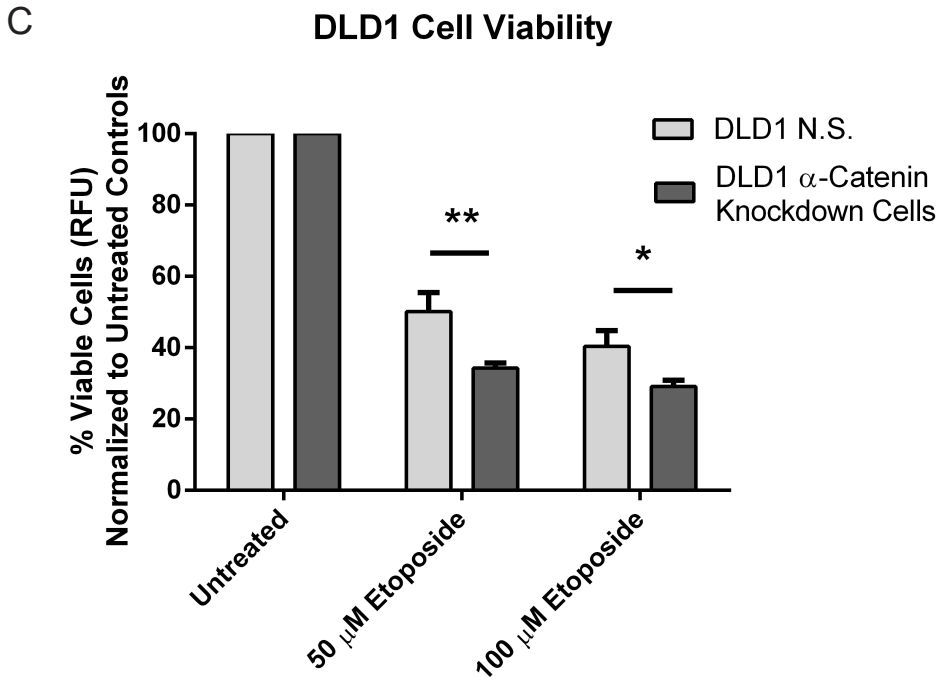
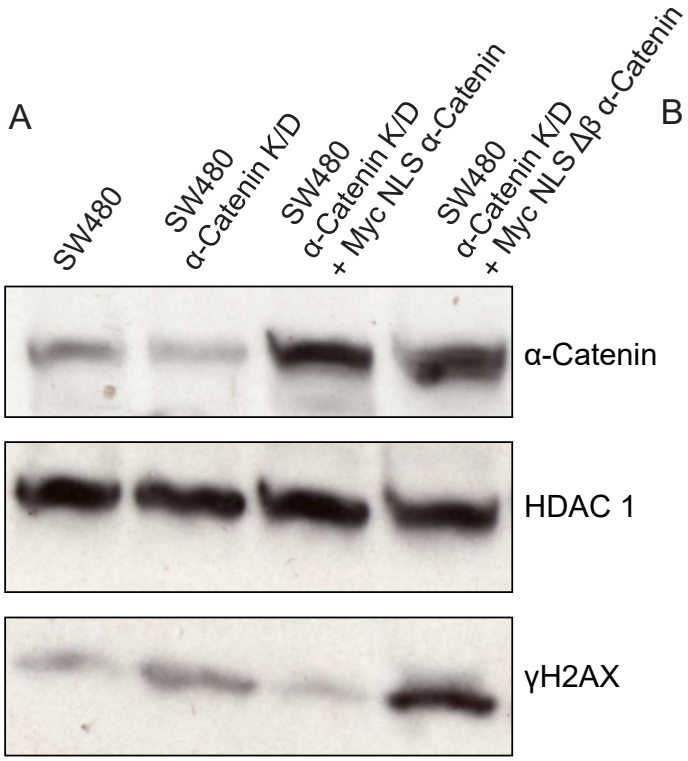


Figure S1: Loss of α-catenin increases γH2AX levels and toxicity in colon cancer cells.

(**A**) DLD1 cells stably transfected with non-specific (N.S.) and α-catenin (α cat K/D) shRNAs were left untreated, treated with 15 μM etoposide for 1 hour, or treated with etoposide for 1 hour and left to recover for 6 hours in fresh medium. Cells were then extracted in hot SDS and Western blots were performed for the indicated proteins. Increased γH2AX levels in etoposide treated α-catenin knockdown cells were observed relative to wild type. (**B**) SW480 cells stably transfected with non-specific (N.S.) and α-catenin (α cat K/D) shRNAs were treated and prepared as in (A). (**C**) DLD1 cells stably transfected with non-specific (N.S.) and α-catenin shRNAs were treated for 48 hours with the indicated concentrations of etoposide. The percentage of cells that remained viable was calculated relative to untreated controls. Mean + SEM is shown (N = 4; * $P < 0.05$, ** $P < 0.01$ by 2-way ANOVA).

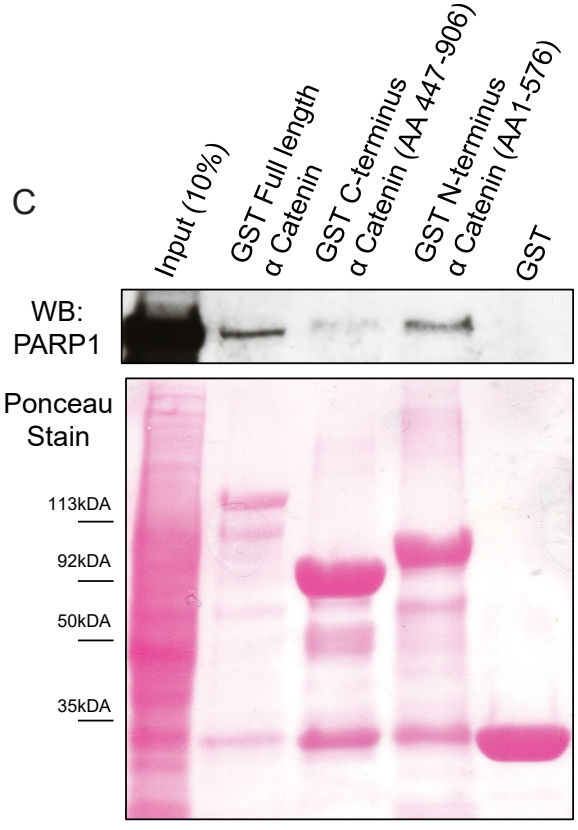
A



B

Identified Proteins	Molecular Weight	Scaffold Identification Probability	Unique # peptides
Catenin alpha-1	100 kDa	100%	69
Glutathione S-transferase P	23 kDa	100%	12
Catenin beta-1	85 kDa	100%	18
ATP-dependent DNA helicase 2 subunit 2 (Ku80)	83 kDa	100%	5
Nucleophosmin	33 kDa	100%	4
ATP-dependent DNA helicase 2 subunit 1 (Ku70)	70 kDa	100%	5
Poly [ADP-ribose] polymerase 1 (PARP1)	113 kDa	100%	4
Nuclease-sensitive element-binding protein 1	36 kDa	100%	2
Catenin alpha-2	105 kDa	100%	2

C



D

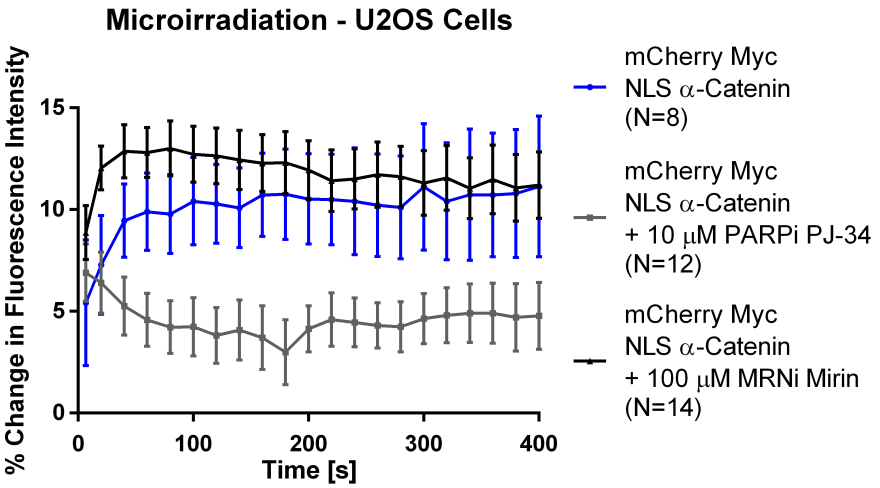
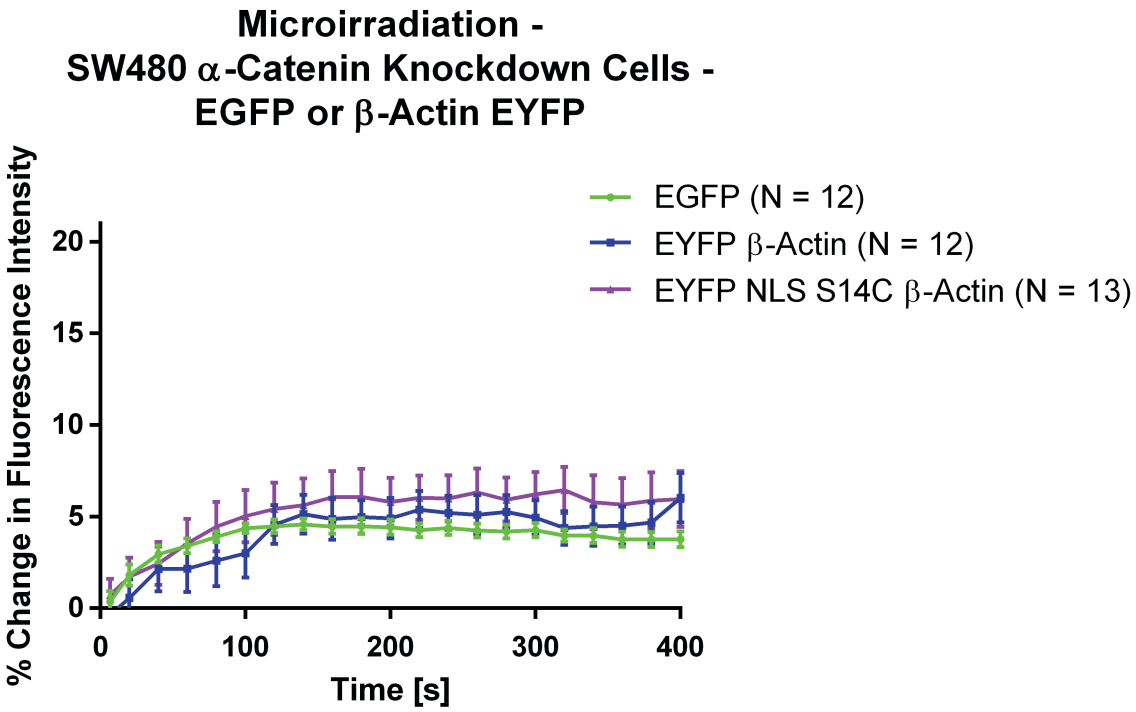


Figure S2: Nuclear α-catenin interacts with several DNA damage proteins.

(**A**) Nuclear extracts isolated from SW480 wildtype cells, α-catenin knockdown cells infected with non-specific hairpin shRNA lentivirus, and α-catenin knockdown cells rescued with lentivirus expressing Myc NLS α-catenin or Myc NLS Δβ α-catenin were blotted for γH2AX. Re-expression of Myc NLS full length α-catenin but not Myc NLS Δβ α-catenin resulted in γH2AX levels similar to wild type cells. HDAC1 was used as a loading control. (**B**) Nuclear extract from SW480 α catenin knockdown cells was incubated with GST α-catenin protein *in vitro* and GST pulldowns were performed. Mass spectrometry analysis identified several DNA damage pathway related proteins. Proteins with a 100% probability of identification and ≥ 2 identified peptides are reported. (**C**) Nuclear extract from SW480 α-catenin knockdown cells was incubated with GST full length α-catenin protein, GST N-terminus α-catenin, GST C-terminus α-catenin, or GST alone *in vitro* and GST pulldowns were performed. Western blotting for PARP1 shows a specific interaction with α-catenin mediated through its N-terminus. Ponceau stain is shown to indicate GST protein levels. (**D**) U2OS cells were transfected with mCherry fused to Myc NLS α-catenin for 48 hours. Cells were then treated with 30 mM LiCl, incubated with Hoechst 33342 (5 μg/mL) to sensitize the cells, and microirradiated to induce localized sites of DNA damage. Recruitment of mCherry Myc NLS α-catenin (N = 8) and mCherry Myc NLS α-catenin pre-treated with the PARP inhibitor, PJ34 (PARPi; N = 12), or Mre11-Rad50-Nbs1 (MRN) complex inhibitor, Mirin, for 1 hour (MRNi; N = 14) are shown (Mean ± SEM). PARP inhibition was able to reduce α-catenin recruitment. However, MRN, which is also responsible for the early recognition of DNA lesions and downstream signaling, was not identified in our proteomics screen and has no effect on α-catenin recruitment.

A



B

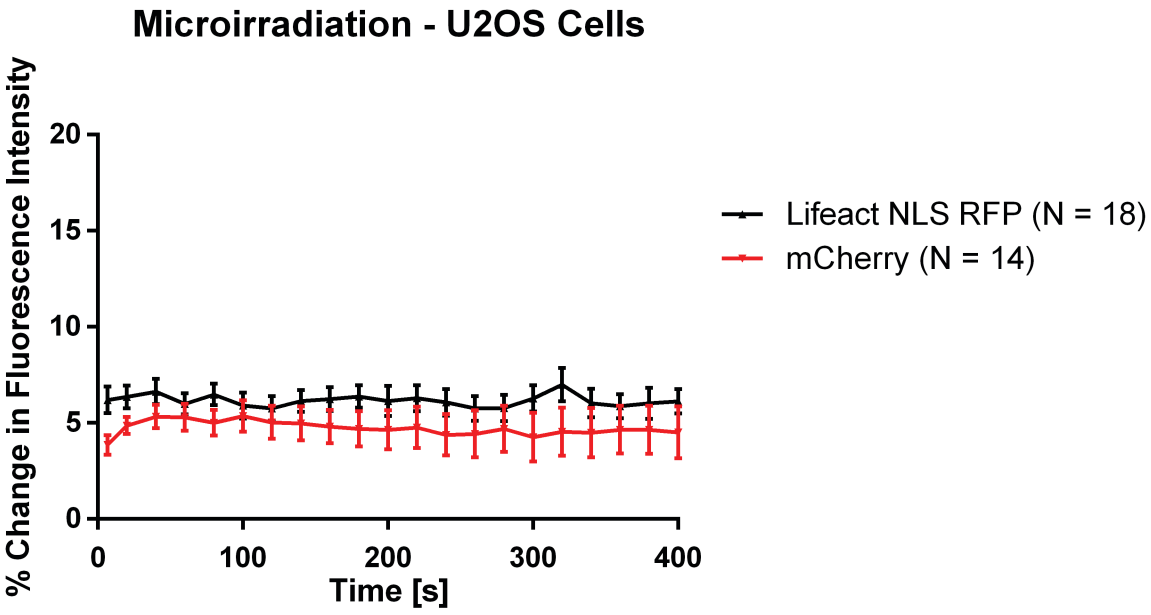


Figure S3: Nuclear actin is not enriched at sites of DNA repair.

(**A**) SW480 α-catenin knockdown cells transfected with EGFP alone, EYFP β-actin, or EYFP NLS S14C β-actin for 48 hours were pre-treated with Hoechst 33342 (5 μg/mL) and microirradiation experiments were performed. Mean ± SEM are shown. (**B**) U2OS cells transfected with mCherry alone or Lifeact NLS RFP for 24 hours were pre-treated with 30 mM LiCl, Hoechst 33342 (5 μg/mL), and microirradiation experiments were performed. Mean ± SEM are shown.