The two SAMP repeats and their phosphorylation state in *Drosophila* Adenomatous polyposis coli-2 play mechanistically distinct roles in negatively regulating Wnt signaling

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ABSTRACT The tumor suppressor Adenomatous polyposis coli (APC) plays a key role in regulating the canonical Wnt signaling pathway as an essential component of the β-catenin destruction complex. C-terminal truncations of APC are strongly implicated in both sporadic and familial forms of colorectal cancer. However, many questions remain as to how these mutations interfere with APC's tumor suppressor activity. One set of motifs frequently lost in these cancer-associated truncations is the SAMP repeats that mediate interactions between APC and Axin. APC proteins in both vertebrates and *Drosophila* contain multiple SAMP repeats that lack high sequence conservation outside of the Axin-binding motif. In this study, we tested the functional redundancy between different SAMPs and how these domains are regulated, using *Drosophila* APC2 and its two SAMP repeats as our model. Consistent with sequence conservation–based predictions, we show that SAMP2 has stronger binding activity to Axin in vitro, but SAMP1 also plays an essential role in the Wnt destruction complex in vivo. In addition, we demonstrate that the phosphorylation of SAMP repeats is a potential mechanism to regulate their activity. Overall our findings support a model in which each SAMP repeat plays a mechanistically distinct role but they cooperate for maximal destruction complex function.

INTRODUCTION

Cooperative actions of various signaling pathways orchestrate proper development of an embryo. Among these pathways, Wnt signaling is an indispensable player, regulating numerous cellular processes, including cell proliferation, migration, and differentiation (Cadigan and Peifer, 2009). Not only is aberrant Wnt signaling detrimental during embryogenesis, but it also leads to numerous diseases in the adult, including various forms of cancer (colon, breast, lung, ovarian, and hepatocarcinoma), metabolic diseases (diabetes and adipogenesis), and neurodegenerative diseases like Alzheimer's disease (Clevers and Nusse, 2012; Inestrosa and Varela-Nallar, 2014; Sherwood, 2015). Thus the tight control of this evolutionarily conserved pathway is essential not only during normal development, but also for adult tissue homeostasis. One of the most crucial steps in controlling this pathway is through negative regulation by the destruction complex or "destructosome," a macromolecular complex whose core components include APC, Axin, and the kinases GSK3 and CK1 (Nusse, 2012). When signaling is off in the absence of a

FIGURE 1: (A) Schematic representation of human APC (hAPC) and APC2 (hAPC2) and *Drosophila* APC1 (dAPC1) and APC2 (dAPC2). Oligomerization domain (burgundy), APC self-association domain (orange), Armadillo (Arm) repeats (blue), 15Rs: 15–amino acid repeats (15Rs) pink; 20–amino acid repeats (20Rs; purple), β-catenin inhibitory domain (yellow), SAMP repeats (shades of red); basic domain (turquoise); EB1 binding domain (magenta); cortical localization domain (C30; dark blue). MCR, mutation cluster region. (B) Sequence alignment of the region that contains the SAMP repeats between human and *Drosophila* APC proteins. Axin-binding motif: hydrophobic motif (yellow) and SAMP motif (red). (C) Yeast two-hybrid experiments demonstrate that both *Drosophila* APC2 SAMP1 and SAMP2 can bind Axin but appear to have different affinities. Empty vector control (orange), Axin-RGS (blue). DB, DNA-binding domain. Error bars represent SD.

Wnt ligand, the destructosome targets the key transcriptional effector of the pathway, β-catenin, for phosphorylation and subsequent proteosome-mediated degradation. Thus loss of any destruction complex component leads to aberrant accumulation of β-catenin, which in turn can drive inappropriate activation of Wnt target genes.

APC is a highly phosphorylated, multidomain protein that can interact with β-catenin via its 15 and 20 amino acid repeats (15Rs and 20Rs; Figure 1A) and with Axin via its SAMP repeats (Figure 1A; Rubinfeld *et al.*, 1995; Behrens *et al.*, 1998). More than 80% of all sporadic and inherited forms of colon cancer carry mutations in the

APC gene (Polakis, 2012). Most of these mutations occur in a region called the mutation cluster region (MCR; Figure 1A), generating APC truncations typically missing some of the 20Rs and all of the SAMP repeats (Beroud, 1996). Over the years, numerous studies have addressed the importance of 15Rs and 20Rs and how phosphorylation of the 20Rs influences the functional interactions with β-catenin (Ha *et al.*, 2004; Xing *et al.*, 2004; Liu *et al.*, 2006). In addition, studies have revealed that there is functional diversity among the 20Rs. For example, the second 20R does not bind β-catenin, due to the absence of an important upstream, charged residue (Liu *et al.*, 2006), but, surprisingly, 20R2 is the only 20R required for APC2 function in the destruction complex (Yamulla *et al.*, 2014). It has been proposed that 20R2 represents a conserved binding site for other protein partners, including potentially the E3 ligase that destroys β-catenin (SCFβ–TrCP; Roberts *et al.*, 2011; Stamos and Weis, 2013), or for Axin (Schneikert *et al.*, 2014). Furthermore, β-catenin binding is not absolutely required for APC's destructosome activity but instead appears to increase the efficiency of β-catenin degradation (Yamulla *et al.*, 2014). Human and *Drosophila* APC proteins each contain multiple SAMP repeats, but it is not known whether these repeats also exhibit functional diversity or whether their activity is regulated by phosphorylation.

Human APC contains three SAMP repeats that are distributed among the 20Rs (Figure 1A). In vitro biochemical studies and the cocrystal structure between Axin's RGS domain and APC's third SAMP repeat mapped the Axin binding to a region containing the conserved motif I/L-L-X-X-C-I-X-S-A-M-P comprising the hydrophobic upstream sequences (hydrophobic motif) and the downstream SAMP motif (Behrens *et al.*, 1998; Spink *et al.*, 2000; Figure 1B). The hydrophobic motif is more highly conserved than the SAMP motif itself, and site-directed mutagenesis of these hydrophobic residues revealed that they are critical for binding Axin (Spink *et al.*, 2000). Germline missense mutations in the hydrophobic motif have also been reported in patients with colorectal cancer, suggesting that these residues could be critical for normal APC function in the destructosome as well (Minde *et al.*, 2011). In contrast, site-directed mutagenesis of the SAMP motif revealed that several changes are compatible with Axin binding (Spink *et al.*, 2000), consistent with the observation that they are less well conserved. Recently, a mechanistic model of the contribution of the SAMP motifs in the destruction complex has been proposed. This model suggests that initial phosphorylation of β-catenin might occur by a SAMP-independent mechanism but that further processing of β-catenin requires Axin oligomerization that is at least partially stimulated through Axin's interactions with the SAMP motifs (Schneikert *et al.*, 2014). Additional evidence indicates that dynamic, GSK3-regulated associations between APC and Axin that are SAMP-independent also exist (Pronobis *et al.*, 2015). Although all APC proteins contain two or more SAMP motifs, functional redundancy or diversity of these motifs has not been explored. However, it has been reported that SAMP1 is sufficient for Wnt regulation in mammals, as the APC1638T truncation containing only SAMP1 is viable and fertile in mice (Smits *et al.*, 1999).

To ask whether functional diversity exists among different SAMP repeats, we used the experimentally tractable *Drosophila* APC2 as our model. Similar to human APC, *Drosophila* APC2 contains more than one SAMP repeat, SAMP1 and SAMP2 (Figure 1A). SAMP2 shares higher sequence conservation to the human homologue within the conserved signature motif, whereas SAMP1 is more degenerate (Figure 1B). In addition to the sequence identity within the Axin-binding motif, both SAMP1 and 2 contain residues that are phosphorylated (Zhai *et al.*, 2008), suggesting a potential regulatory

mechanism that has not been previously explored. These observations may suggest that there is functional divergence between different SAMP repeats.

Here we test for the first time the functional importance of different SAMP repeats in *Drosophila* APC2. We demonstrate that rather than being redundant as once predicted, they are distinct in their ability to interact with Axin, their effect on destructosome activity, and the extent to which phosphorylation is important for their regulation. Although SAMP1 is surprisingly sufficient for β-catenin degradation in human SW480 cells, both SAMPs are essential for destruction function in the *Drosophila* embryo. Taken together, our data suggest that individual SAMP repeats have distinct yet cooperative activities.

RESULTS

Both SAMP1 and SAMP2 of *Drosophila* APC2 can bind Axin but with different strengths

To gain insight into the APC–Axin interaction and the importance of individual residues within the Axin-binding motif, we examined the level of sequence conservation of the Axin-binding motif across diverse vertebrate and invertebrate taxa (Supplemental Figure S1A). Although the SAMP motif itself (Supplemental Figure S1A, red) shows some sequence diversity between vertebrate and invertebrate APCs and between APC and APC2 proteins, the upstream hydrophobic residues (Supplemental Figure S1A, yellow) are well conserved, with the exception of vertebrate APC2s. The higher evolutionary pressure to retain these residues compared with the SAMP motif itself suggests that these residues play a more significant role in Axin binding or some other aspect of destructosome function. This is consistent with analysis of the APC–Axin crystal structure (Spink *et al.*, 2000) and might explain why germline missense mutations in the upstream hydrophobic residues can be found in some cases of colorectal cancer patients (Minde *et al.*, 2011), although we have not identified SAMP motif mutants in the literature.

Drosophila APC2 contains two SAMP repeats, SAMP1 and SAMP2. SAMP2 contains a conserved hydrophobic motif but partially conserved SAMP motif compared with the mammalian APCs; however, compensatory mutations in *Drosophila* Axin may allow for efficient repacking in this area (Spink *et al.*, 2000; Figure 1B). Similarly, SAMP1 contains the conserved upstream hydrophobic motif, but the SAMP motif is not conserved. This suggested that SAMP1 might not participate in Axin binding and destruction complex function and prompted us to test the activities of these repeats individually, first by asking whether the individual SAMP repeats interact with Axin, using a yeast two-hybrid (Y2H) assay.

APC2 containing both SAMPs (APC2-Cterm-SAMP) can interact with the APC-binding domain of Axin (Axin-RGS; Figure 1C). Consistent with our hypothesis that SAMP1 is a weak Axin binder, APC2-C-term-SAMP1 displayed a significantly weaker interaction with Axin than with APC2-C-term-SAMP2 (Figure 1C). Of interest, we observed the strongest binding interaction when both SAMP repeats were present (APC2-Cterm-SAMP), suggesting an additive or cooperative effect. Taken together, these results suggest that SAMP2 is the primary Axin-binding SAMP in APC2, and thus we predicted that SAMP2 would have the greater role in destructosome function.

Either SAMP is sufficient to recruit APC2 to the destructosome in *Drosophila* S2 cells

Overexpressed or endogenous Axin in cell culture and intact tissues forms cytoplasmic puncta due in part to its ability to form oligomers (Fagotto *et al.*, 1999; Faux *et al.*, 2008; Fiedler *et al.*, 2011). In *Drosophila* cultured S2 cells, APC2 is recruited into these puncta via
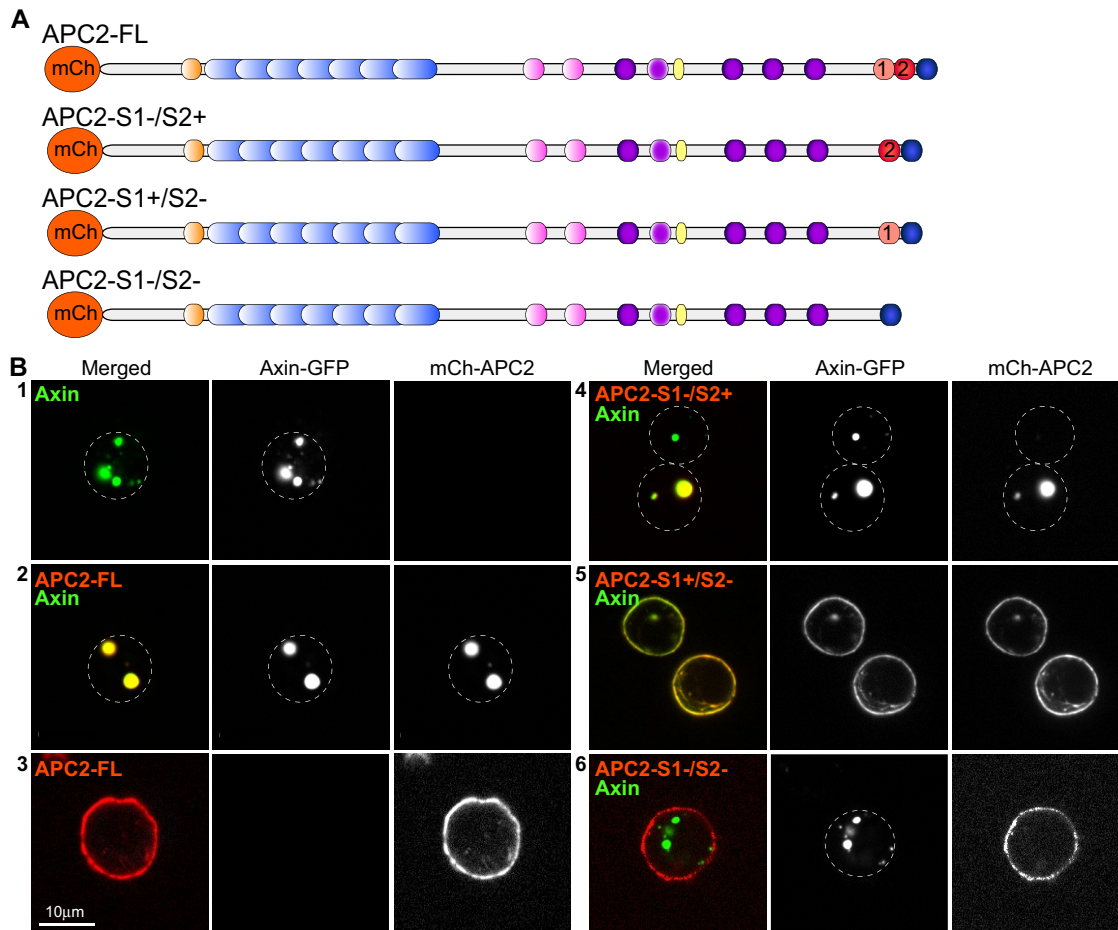
FIGURE 2: Either SAMP repeat is sufficient for the recruitment of APC2 to the destructosome puncta. (A) Schematic representation of mCherry (mCh)-tagged APC2 constructs. Full-length APC2: APC2-FL; APC2 lacking only SAMP1: APC2-S1–/S2+; APC2 lacking only SAMP2: APC2-S1+/S2–; APC2 lacking both SAMP1 and SAMP2: APC2-S1–/S2–. (B) GFP-tagged Axin forms oligomers, which can be visualized as cytoplasmic puncta (1). When coexpressed, mCh-APC2-FL colocalizes in cytoplasmic puncta with Axin-GFP (2), but mCh-APC2-FL expressed without extra Axin is primarily cortical (3). Removal of either of APC2's SAMP domains (APC2-S1–/S2+ [4] and APC2-S1+/S2– [5]) does not affect colocalization with Axin-GFP, but APC2-S1+/S2– relocates Axin to cortex. Removal of both SAMPs (APC2-S1–/S2–) prevents APC2–Axin colocalization (6). Dotted lines indicate cell boundaries. Scale bar, 10 μm.

its SAMP repeats (Kunttas-Tatli *et al.*, 2014; and compare Figure 2B, 2 to 6), although APC and Axin can interact via SAMP-independent mechanisms as well (Roberts *et al.*, 2011; Schneikert *et al.*, 2014). We used this system to ask whether the differences in Axin binding we observed by Y2H affected APC2's ability to be recruited to the destructosome by Axin. To determine whether APC2 SAMP1 or SAMP2 has a more significant role in destructosome localization, we generated mutants that lacked SAMP1 (APC2-S1–/S2+), SAMP2 (APC2-S1+/S2–), or both (APC2-S1–/S2–; Figure 2A). While generating these deletion mutants, we retained the last 30 amino acids (C30) because this sequence is required for the cortical localization of *Drosophila* APC2 (Zhou *et al.*, 2011). We coexpressed the APC2 SAMP mutants (APC2-S1–/S2+ and APC2-S1+/S2–) with Axin–green fluorescent protein (GFP; Figure 2B, 4 and 5). Whereas both SAMPs have the ability to bind Axin by Y2H (Figure 1C), we predicted that the weaker binder, SAMP1, would have reduced ability to facilitate recruitment. However, we observed that either SAMP repeat was sufficient for destructosome recruitment. Surprisingly, expression of APC2-S1+/S2– (lacking the stronger Axin binder, SAMP2) resulted in redistribution of Axin to the cell cortex in a proportion of the cells

(Supplemental Figure S2A, 1–3). Because the ability of Axin to form puncta might depend on its expression level, we used fluorescence-activated cell sorting (FACS) to sort the cells on the basis of their Axin expression levels. This revealed that Axin redistribution to the cortex in the presence of APC2 was enhanced in cells expressing high levels of Axin-GFP (Supplemental Figure S2B).

We also tested whether both SAMPs were able to colocalize with Axin in SW480 human colon cancer cells. Similar to *Drosophila* S2 cells, either SAMP repeat was sufficient for colocalization with Axin (Figure 3A). However, unlike S2 cells, we did not observe redistribution of Axin to the cortex in APC2-S1+/S2–. This is consistent with the fact that normal distribution of APC2 is not cortical in SW480 cells (Roberts *et al.*, 2011), possibly due to the absence of a cortical binding partner.

SAMP1, but not SAMP2, is necessary for destructosome activity in SW480 cells

Because SAMP2 is a stronger Axin binder by Y2H, we predicted that it would be the primary SAMP required for destructosome activity. To test this, we asked whether SAMP2 is necessary and/or sufficient
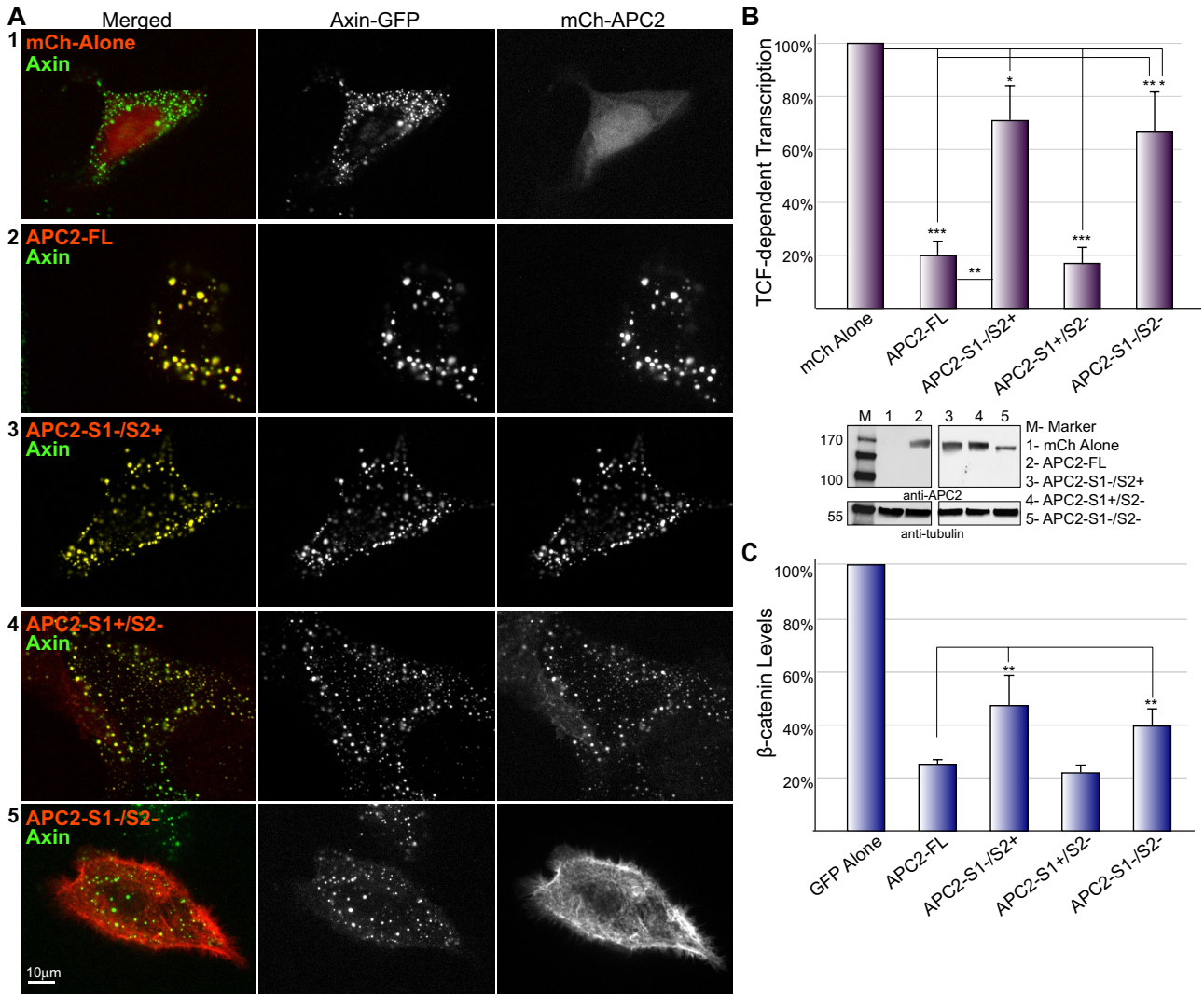
FIGURE 3: SW480 cells reveal differential requirements for SAMP1 and SAMP2 in β-catenin regulation. (A) Similar to S2 cells, Axin-GFP forms cytoplasmic puncta in SW480 cells (1), and mCh-APC2 colocalizes with Axin-GFP in those puncta (2). Either SAMP is sufficient for colocalization with Axin-GFP in cytoplasmic puncta (3, 4), but removal of both domains disrupts colocalization (5). Scale bar, 10 µm. (B) TOPFlash assays demonstrated that the expression of full-length *Drosophila* APC2 (APC2-FL) is sufficient to suppress the elevated levels of TCF-dependent transcriptional activity in SW480 cells. APC2-S1+/S2– had activity indistinguishable from that of APC2-FL. In contrast, deletion of either SAMP1 (APC2-S1–/S2+) or both SAMPs (APC2-S1–/S2–) resulted in a significant loss of APC2 activity. The Western blot demonstrates that the mCh-tagged APC2-FL and all the SAMP deletion constructs were expressed at comparable levels in SW480 cells used in the TOPFlash assays. (C) Similar to the TOPFlash assays, expression of APC2-FL or APC2-S1+/S2– was sufficient to suppress the elevated levels of β-catenin, whereas APC2-S1–/S2+ and APC2-S1–/S2– exhibited a significant loss of β-catenin destruction function. Error bars represent SD. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$.

for APC2's destructosome function. Human SW480 cells endogenously express an APC MCR truncation that leads to elevated levels of β-catenin and high levels of Wnt target gene expression due to loss of destructosome activity (Munemitsu *et al.*, 1995). Previous studies showed that expression of *Drosophila* APC2 could compensate for the loss of hAPC function to suppress the elevated levels of β-catenin and the high levels of Wnt target gene expression (Roberts *et al.*, 2011, 2012; Yamulla *et al.*, 2014). β-Catenin–mediated transcriptional activity is measured by the well-established TOPFlash luciferase assay (Korinek *et al.*, 1997). We predicted that APC2-S1+/S2– would have strongly reduced APC activity and APC2-S1–/S2+ would be sufficient to promote β-catenin destruction. Surprisingly, APC2-S1+/S2– functioned as well as APC2-FL (full length), whereas

APC2-S1–/S2+ behaved like the complete SAMP deletion (Figure 3B). As a control, we examined the expression levels of the transfected constructs; all APC2 mutants were expressed at similar levels (Figure 3B, bottom). The lower level of APC2-S1–/S2– protein may be attributed to decreased protein stability due to its lack of destructosome localization. We also measured β-catenin levels in FACS-sorted SW480 cells. Consistent with the TOPFlash results, APC2-S1+/S2– was similar to the full length, whereas both APC2-S1–/S2+ and APC2-S1–/S2– exhibited reduced β-catenin degradation (Figure 3C). Taken together, these data suggest that the weaker Axin binder, SAMP1, plays a functionally significant role in destructosome activity, whereas the stronger Axin binder, SAMP2, is dispensable in SW480 cells.

SAMP1 and SAMP2 are both essential for destructosome activity in the *Drosophila* embryo

Between 4 and 6 h after egg laying, Wnt signaling plays a key role in the patterning of embryonic segments in *Drosophila*. Whereas ventral epidermal cells receiving Wnt secrete a smooth cuticle, ventral cells not receiving the signal generate cuticular projections called denticles (arrows in Figure 4C). The same pattern can be visualized as an accumulation of Armadillo (Arm; fly ß-catenin) in stripes of cells receiving Wnt signal and in the patterned expression of the Wnt target gene *engrailed* (Figure 4D1). This pattern is disrupted in embryos with aberrant Wnt signaling such as in APC mutants. In *Drosophila*, APC1 and APC2 play redundant roles in Wnt signaling throughout development. *APC1*-null (*APC1^{Q8}*) mutant flies are adult viable, with phenotypes restricted to apoptosis of photoreceptors in the compound eye (Ahmed *et al.*, 2002). In contrast, *APC2*-null (*APC2^{g10}*) embryos are embryonic lethal, displaying a modest accumulation of Arm, an expanded *engrailed* expression domain, and a significant loss of denticles (McCartney *et al.*, 1999, 2006; Kunttas-Tatli *et al.*, 2012); Supplemental Figure S4, A and D2). Although APC2 is the primary Wnt regulator during embryogenesis, APC1 does contribute destructosome function; loss of *APC1* in the embryo enhances the *APC2*-null phenotype with a more robust accumulation of Arm and complete loss of denticles (Ahmed *et al.*, 2002; Akong *et al.*, 2002). Previous studies suggested that testing *APC2* mutants in both the *APC2* single-null and *APC2 APC1* double-null backgrounds is informative: the *APC2 APC1* double-null background provides the most stringent test of destruction function, requiring substantial transgene function for rescue, whereas the *APC2* single-null background can reveal more subtle differences (Roberts *et al.*, 2011; Kunttas-Tatli *et al.*, 2012).

To examine the role of the individual SAMP repeats in a more physiologically relevant context, we first expressed GFP-tagged APC2-FL and the SAMP deletion mutants (APC2-S1–/S2+, APC2-S1+/S2–, APC2-S1–/S2–) in *APC2* single-null embryos under the native *APC2* promoter (McCartney *et al.*, 2006). All transgenes were expressed at a level comparable to endogenous APC2 (Supplemental Figure S3, B and C), and neither the SAMP deletions nor the phosphomutations disrupted proper cortical localization in vivo (Supplemental Figure S3A). Of interest, the APC2-S1+/S2– mutant appeared to localize to the embryonic cortex more strongly than wild-type APC2 or the other mutants, consistent with our S2 cell experiments (Supplemental Figure 2B5). As demonstrated previously, GFP-APC2-FL fully rescued the *APC2*-null phenotype in the embryo (Roberts *et al.*, 2011; Kunttas-Tatli *et al.*, 2012; Supplemental Figure S4, A and D3). Consistent with the TOPFlash analysis (Figure 3B), and previous reports (Roberts *et al.*, 2011), APC2-S1–/S2– had significantly reduced destructosome function; removal of both SAMPs resulted in only a 36% hatch rate, moderate cuticle defects, and elevated Arm levels sufficient to result in the expansion of *en* expression (Supplemental Figure S4, A, B, and D6). This suggests that the SAMP repeats are necessary for APC2's destructosome activity. On the basis of the TOPFlash assay (Figure 3B), we predicted that SAMP1, but not SAMP2, would be necessary and sufficient for destructosome function. Surprisingly, we found that both APC2-S1+/S2– and APC2-S1–/S2+ were able to completely rescue the *APC2*-null defects (Supplemental Figure S4, A and B), indicating that either SAMP1 or SAMP2 is sufficient for function in the *APC2* single-null background.

To assess potential redundancy of the SAMPs in a more stringent background, we also tested our *APC2* SAMP mutants in the *APC2 APC1* double-null background. *APC2 APC1* mutant embryos were generated using the standard FRT/FLP/DFS method (Chou and Perrimon, 1996), in which 50% of the embryos are maternally and zygotically null for *APC1* and *APC2* (MZ class) and 50% are maternally null but zygotically heterozygous due to paternal contribution (MZ+ class; Supplemental Figure S4C). The MZ and MZ+ classes can be distinguished by assessing the embryonic hatch rates and cuticle phenotypes. In the absence of any transgene, nearly 11% of the embryos hatched (MZ+), and of those that died, 30% displayed weak cuticle defects (ranging from 0 to 2.5; MZ+), whereas the remaining progeny displayed strong cuticle defects (ranging from three to six; MZ; Figure 4, A and B). Expression of the full-length transgene (APC2-FL) in this double-mutant background increased the hatch rate (37% of total progeny) and strongly suppressed the cuticle defects in those MZ and MZ+ progeny that die as embryos (Figure 4, A–C). Expression of APC2-S1–/S2– in the double mutant failed to rescue the Wnt pathway activation defects. In fact, APC2-S1–/S2– exerted a dominant-negative effect, as evidenced by the lower hatch rate (<1%) and the strong cuticle defects exhibited by all of the lethal progeny (Figure 4, A–C). This dominant-negative effect was previously observed for deletion of both SAMPs (Roberts *et al.*, 2011). APC2-S1–/S2+, missing SAMP1 and retaining SAMP2, provided only very weak destruction activity, evidenced by a decrease in the percentage of embryos with strong cuticle defects and a comparable increase in the percentage of embryos with weak cuticle defects. In contrast, APC2-S1+/S2– exhibited dominant-negative activity similar to, but stronger than, APC2-S1–/S2– (Figure 4, A–C). These results suggest that both SAMP1 and SAMP2 are necessary for APC2's destructosome function and that neither alone is sufficient. Furthermore, the loss of SAMP2, in the presence or absence of SAMP1, produces a protein with toxic effects on any remaining destructosome function that exists in the absence of APC2 activity. This finding is in striking contrast to the SW480 results indicating that SAMP2 is dispensable and SAMP1 is both necessary and sufficient for destruction function (Figure 3B).

Disrupting SAMP1 or SAMP2 phosphorylation has no effect on the localization of APC2 to destructosome puncta with Axin

APC proteins are regulated via phosphorylation in many contexts. A well-studied example is the multiphosphorylation of the 20Rs by CK1 and GSK3β (Rubinfeld *et al.*, 1996). Phosphorylation of 20Rs enhances β-catenin binding affinity (Ha *et al.*, 2004; Xing *et al.*, 2004; Liu *et al.*, 2006), and either blocking or mimicking phosphorylation in a subset of 20Rs significantly impairs APC2's destructosome activity in vivo (Kunttas-Tatli *et al.*, 2012). Mass spectrometry (MS) analysis of proteins expressed during *Drosophila* embryogenesis revealed multiple sites in the APC2 SAMP repeats that are phosphorylated (Zhai *et al.*, 2008). By combining the MS data with additional computational predictions, we hypothesized that eight sites in SAMP1 and eight sites in SAMP2 may be phosphorylated, potentially suggesting a regulatory mechanism for APC2's destructosome function (Figure 5A). To test this hypothesis, we generated targeted mutations in the APC2 SAMP repeats that prevent phosphorylation at these sites: APC2-S1^{SA}/S2+, APC2-S1/S2^{SA}, and APC2-S1^{SA}/S2^{SA} (Figure 5A).

To determine whether disruption of phosphorylation had an effect on recruitment of APC2 to Axin puncta or on puncta assembly, we coexpressed the APC2 phosphodeficient SAMP mutants with Axin-GFP first in S2 cells. Disruption of phosphorylation had no detectable effect on colocalization with Axin. However, similar to the expression of APC2-S1+/S2– (Figure 2B), blocking phosphorylation in SAMP1 (APC2-S1^{SA}/S2+) or in both SAMPs (APC2-S1^{SA}/S2^{SA}) resulted in redistribution of Axin to the cell cortex in a proportion of the cells (Figure 5B, 3 and 5, and Supplemental Figure S2, A, 4–6, and B).
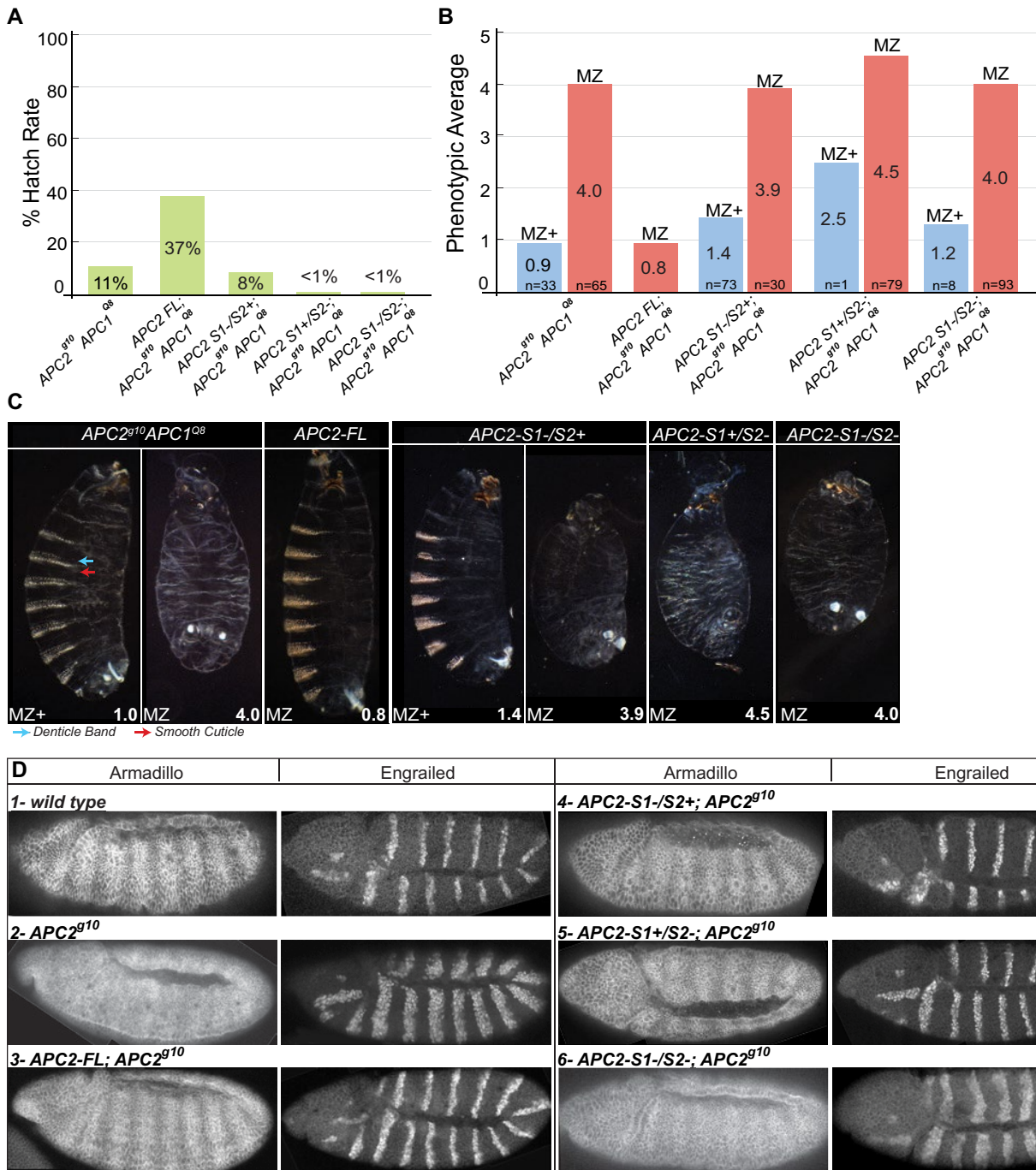
FIGURE 4: Both SAMP1 and SAMP2 are necessary for destructosome activity in the *Drosophila* embryo. (A, B) Hatch rate and cuticle analysis of *APC2 APC1* double-null (*APC2^{g10}APC1^{Q8}*) embryos with transgenes expressing APC2-FL and SAMP deletion mutants. (B, C) The cuticle phenotypes of embryos that failed to hatch were assessed, and the phenotypic average (PA) was calculated for mutant embryos. Cuticles were classified as either MZ+ (maternally mutant but zygotically rescued) or MZ (maternally and zygotically mutant). Higher numbers indicate more severe defects (scoring criteria as in McCartney *et al.*, 2006). *N*, number of embryos scored. (D) Representative embryos showing the striped Arm accumulation and En expression. In all images, anterior is to the left and dorsal is up. Differential Arm accumulation is lost and the En expression domain is expanded in *APC2*-null (*APC2^{g10}*) embryos (2) compared with wild type (1). APC2-FL (3) and either single SAMP deletion (APC2-S1–/S2+ [4] and APC2-S1–/S2+ [5]) are sufficient to restore the normal Arm and En patterns in *APC2*-null embryos. In contrast, deletion of both SAMPs (APC2-S1–/S2–) (6) fails to rescue the wild-type pattern of Arm and En. Scale bar, 25 μm.

SAMP1 phosphorylation, but not SAMP2 phosphorylation, is necessary for destructosome activity in SW480 cells

Consistent with *Drosophila* S2 cells, disruption of neither SAMP1 nor SAMP2 phosphorylation had an effect on recruitment of APC2 to the destructosome in SW480 cells (Figure 6A). Similar to complete loss of SAMP1 (APC2-S1–/S2+; Figure 3B), disrupting SAMP1 phosphorylation (APC2-S1^{SA}/S2+) or all SAMP phosphorylation (APC2-S1^{SA}/S2^{SA}) resulted in proteins with reduced destructosome
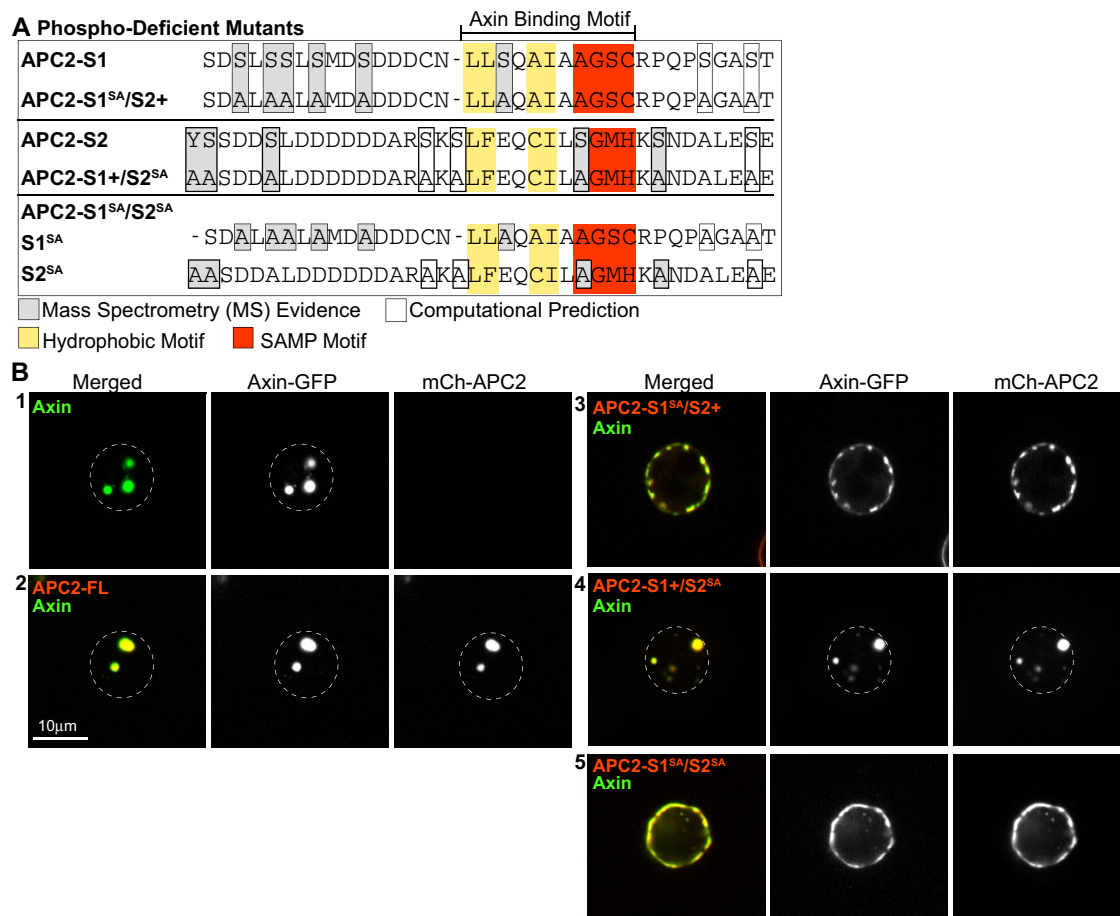
FIGURE 5: APC2-SAMP phosphomutants all colocalize with Axin but some relocate Axin to the cortex. (A) Sequences of APC2 SAMP phosphodeficient constructs. The Axin-binding motif is indicated in yellow and orange. Residues in gray and white boxes in the wild-type sequences are predicted phosphorylation sites (mass spectrometry evidence or computational prediction, respectively). (B) All SAMP mutants colocalize with Axin (3–5) as with the FL protein (2); however, APC2-S1^{SA}/S2+ (3) and APC2-S1^{SA}/S2^{SA} (5) redistribute Axin to the cortex (similar to APC2-S1+/S2–). Dotted lines indicate cell boundaries. Scale bar, 10 μm.

function in the TOPFlash assay (Figure 6B). As a control, we measured the expression levels of the constructs; all APC2 mutants were expressed at comparable levels (Figure 6B, bottom). In contrast, APC2-S1+/S2^{SA} fully suppressed the elevated levels of Wnt target gene activity similar to APC2-FL and APC2-S1+/S2– (Figures 3B and 6B). To complement the TOPFlash experiments, we also measured levels of β-catenin in FACS-sorted SW480 cells. In this case, disrupting phosphorylation in either SAMP1 or SAMP2 (APC2-S1^{SA}/S2+ and APC2-S1+/S2^{SA}) led to behavior similar to APC2-FL, whereas only APC2-S1^{SA}/S2^{SA} displayed a significant difference in promoting efficient β-catenin destruction (Figure 6C). This suggests that small differences in β-catenin levels can affect transcriptional output (Roberts *et al.*, 2011). Taken together, these results indicate that SAMP2 phosphorylation is not necessary for the activity of the destructosome in SW480 cells, but phosphorylation of SAMP1 is required for optimal destruction function.

Phosphorylation of both SAMP1 and SAMP2 is necessary for destructosome activity in the *Drosophila* embryo

On the basis of the TOPFlash assay (Figure 6B), we predicted that SAMP1 phosphorylation, but not SAMP2 phosphorylation, would be necessary for destructosome function in vivo. To test this hypothesis, we again expressed these APC2 mutants in the *APC2*

single-null and *APC2 APC1* double-null backgrounds. Disrupting phosphorylation in either SAMP alone (APC2-S1^{SA}/S2+ or APC2-S1+/S2^{SA}) had no effect on APC2 function in *APC2*-null embryos (Supplemental Figure S4, A and B), similar to the deletion mutants (Figure 3A). However, disrupting phosphorylation in both SAMPs (APC2-S1^{SA}/S2^{SA}) significantly reduced APC2 function (71% hatch rate, moderate cuticle defects, elevated Arm levels, and expanded *en* expression; Supplemental Figure S4, A and B, and Figure 7D) but not as severely as complete deletion of the SAMPs (Supplemental Figure S4, A and B). This suggests that SAMP repeat phosphorylation is necessary for APC2's destructosome activity.

Similar to the deletion mutants, we tested the destructosome function of the phosphodeficient SAMP mutants in the *APC2 APC1* double-null background, and with this more sensitive assay, the significance of SAMP phosphorylation became clear. Phosphodeficient SAMP1 mutant alone (APC2-S1^{SA}/S2+) completely eliminated APC2 activity (Figure 7, A and B), whereas phosphodeficient SAMP2 mutant (APC2-S1+/S2^{SA}) resulted in a largely functional APC2 protein, as evidenced by a higher hatch rate and weaker cuticle phenotypes (Figure 7, A and B). Surprisingly, APC2 without any potential SAMP phosphorylation (APC2-S1^{SA}/S2^{SA}) also has partial function, as its hatch rate and cuticle phenotypes more closely matched those of the SAMP2 phosphorylation mutant (Figure 7, A and B). Collectively
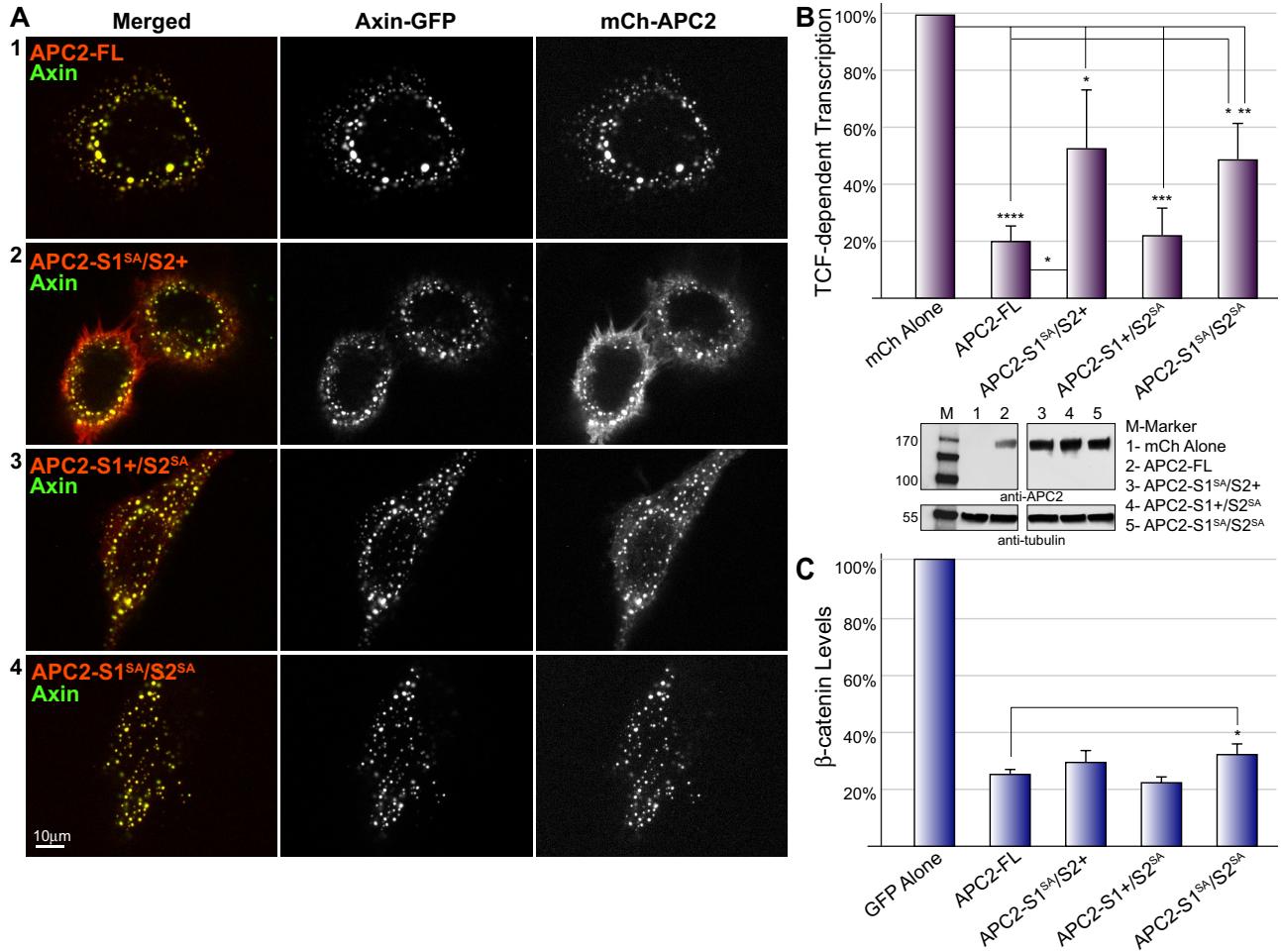
FIGURE 6: SW480 cells reveal differential requirements for phosphorylation of SAMP1 and SAMP2 in β-catenin regulation. (A) All forms of APC2 colocalize with Axin-GFP in cytoplasmic puncta. (B) Both FL-APC2 and APC2-S1+/S2^{SA} were sufficient to suppress high levels of target gene activity in the TOPFlash assay. In contrast, disruption of phosphorylation in SAMP1 alone (APC2-S1^{SA}/S2+) or in both SAMPs (APC2-S1^{SA}/S2^{SA}) reduced APC2's activity. mCh-tagged APC2-FL and all of the phosphodeficient SAMP mutants were expressed at comparable levels in the SW480 cells used in the TOPFlash assays. (C) Surprisingly, both single SAMP phosphomutants were able to suppress high levels of β-catenin accumulation in SW480 cells, like APC2-FL. β-Catenin only accumulated in cells expressing APC2-S1^{SA}/S2^{SA}, and then only mildly. Error bars represent SD. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$.

these findings reveal a surprising interplay between phosphorylation of the SAMP motifs. SAMP1 phosphorylation is required for APC2 destructosome activity, but the observation that blocking SAMP2 phosphorylation mitigates the effect of loss of SAMP1 phosphorylation suggests that there is mechanistic cross-talk between the two SAMP repeats that is mediated by phosphorylation.

Conserved upstream phosphoresidues in SAMP1 are sufficient for SAMP1 function in the destructosome

Despite the lack of sequence conservation in the SAMP motif itself, the upstream phosphoresidues in APC2-SAMP1 are highly conserved in both vertebrate and most invertebrate APC-SAMP3s, which contain putative phosphorylation sites for CK1 and GSK3 (Supplemental Figure S1, B and C). Therefore we predicted that the upstream phosphoresidues would play the more significant role in SAMP1's destructosome function. To test this hypothesis, we generated mutants that disrupted (APC2-S1^{SAup}/S2+) or mimicked (APC2-S1^{SDup}/S2+) phosphorylation only in these residues. As a complement, we also generated a mutant that disrupts only the downstream

putative phosphoresidues (APC2-S1^{SAdown}/S2+; Figure 8). Similar to our results with the behavior of APC2-S1^{SA}/S2+, none of these mutants affected colocalization with Axin or the formation of puncta in S2 cells (Figure 8B). Of interest, none of these mutants drove Axin to the cortex as we observed with APC2-S1^{SA}/S2+ (Figure 8B), suggesting that this effect requires both the upstream and downstream putative phosphoresidues.

Because TOPFlash in SW480 cells consistently demonstrated the role of SAMP1 in destruction function (Figures 3 and 6), we used this assay alone to assess the destructosome function of these mutants. Whereas the SAMP1 mutant that mimics phosphorylation at the upstream sites (APC2-S1^{SDup}/S2+) behaved similar to full length, blocking upstream SAMP1 phosphorylation (APC2-S1^{SAup}/S2+) mildly reduced destructosome function in SW480 cells (Figure 8C). Surprisingly, the mutant with wild-type upstream residues but disrupted downstream residues (APC2-S1^{SAdown}/S2+) displayed destructosome activity that was even more efficient than that of APC2-FL (Figure 8C), suggesting that the ability of both the upstream and the downstream sequences to be phosphorylated is important for
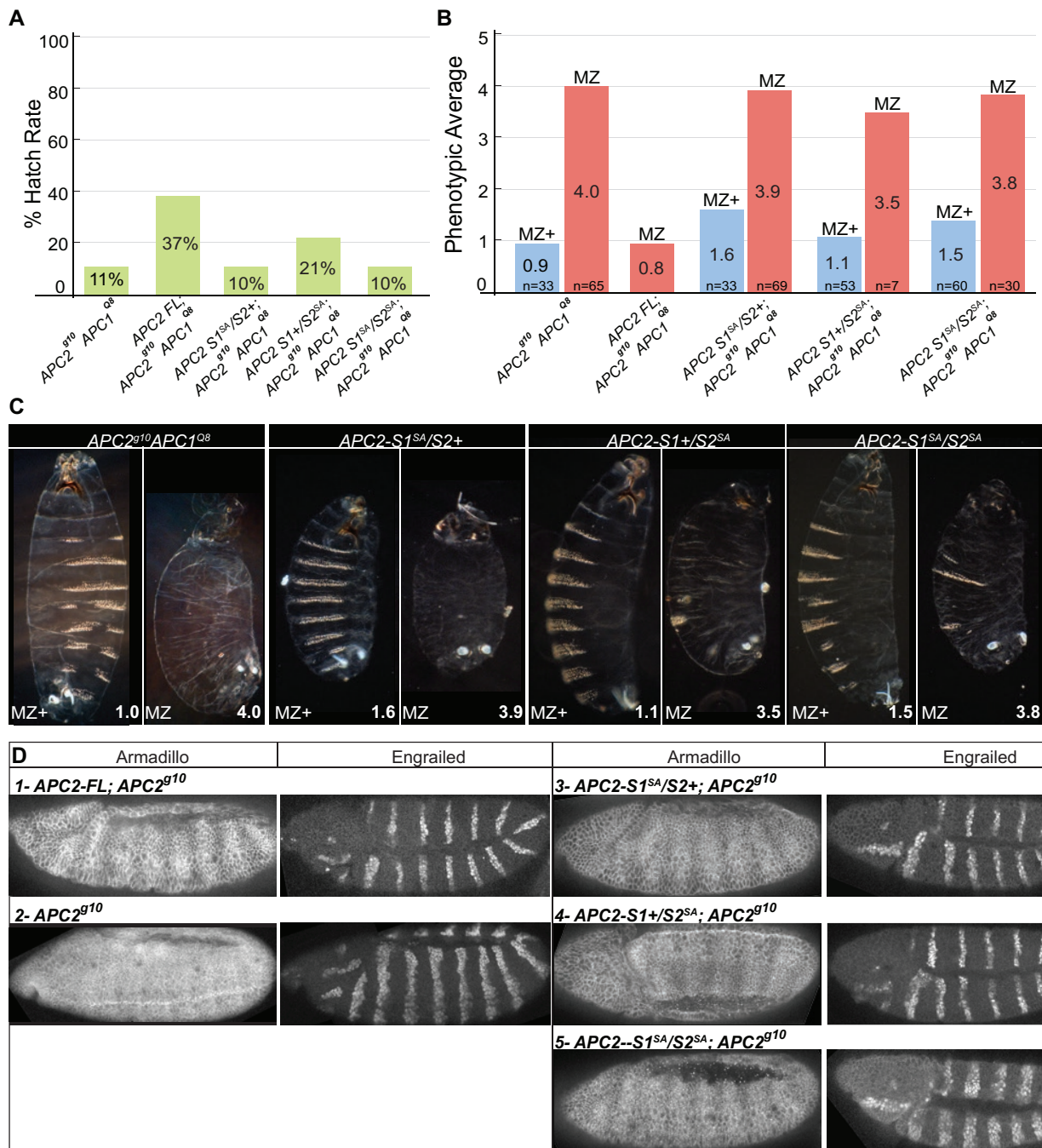
FIGURE 7: Phosphorylation of both SAMP1 and SAMP2 plays important roles in destructosome activity in the *Drosophila* embryo. (A, B) Hatch rate and cuticle analysis of *APC2 APC1* double-null (*APC2^g10^APC1^Q8^*) embryos with transgenes expressing APC2-FL and SAMP phosphomutants. (B, C) The cuticle phenotypes of embryos that failed to hatch were assessed, and the phenotypic average (PA) was calculated for mutant embryos. Cuticles were classified as either MZ+ (maternally mutant but zygotically rescued) or MZ (maternally and zygotically mutant). Cuticle images are shown at the same scale. (D) In the single–*APC2* null mutant, only APC2-S1^SA^/S2^SA^ appears to have reduced function based on the accumulation of Arm and the expression domain of En. Scale bar, 25 μm.

APC2's destructosome function. This suggests that in the absence of SAMP1 upstream phosphorylation, other mechanisms, such as downstream phosphorylation, may partially support APC2's destructosome activity.

DISCUSSION

Despite significant interest in understanding the role of APC proteins in the regulation of Wnt signaling, there are outstanding questions regarding the fundamental molecular mechanisms of its destructosome activity. Previous studies focusing on APC's 20Rs showed that they display functional diversity and that phosphorylation of at least a subset of 20Rs plays an important role in APC's destructosome activity (Roberts *et al.*, 2011; Kunttas-Tatli *et al.*, 2012), although direct binding of β-catenin to APC does not appear essential for targeting β-catenin for destruction (Yamulla *et al.*, 2014). In this study, we focused on the SAMP repeats, the known
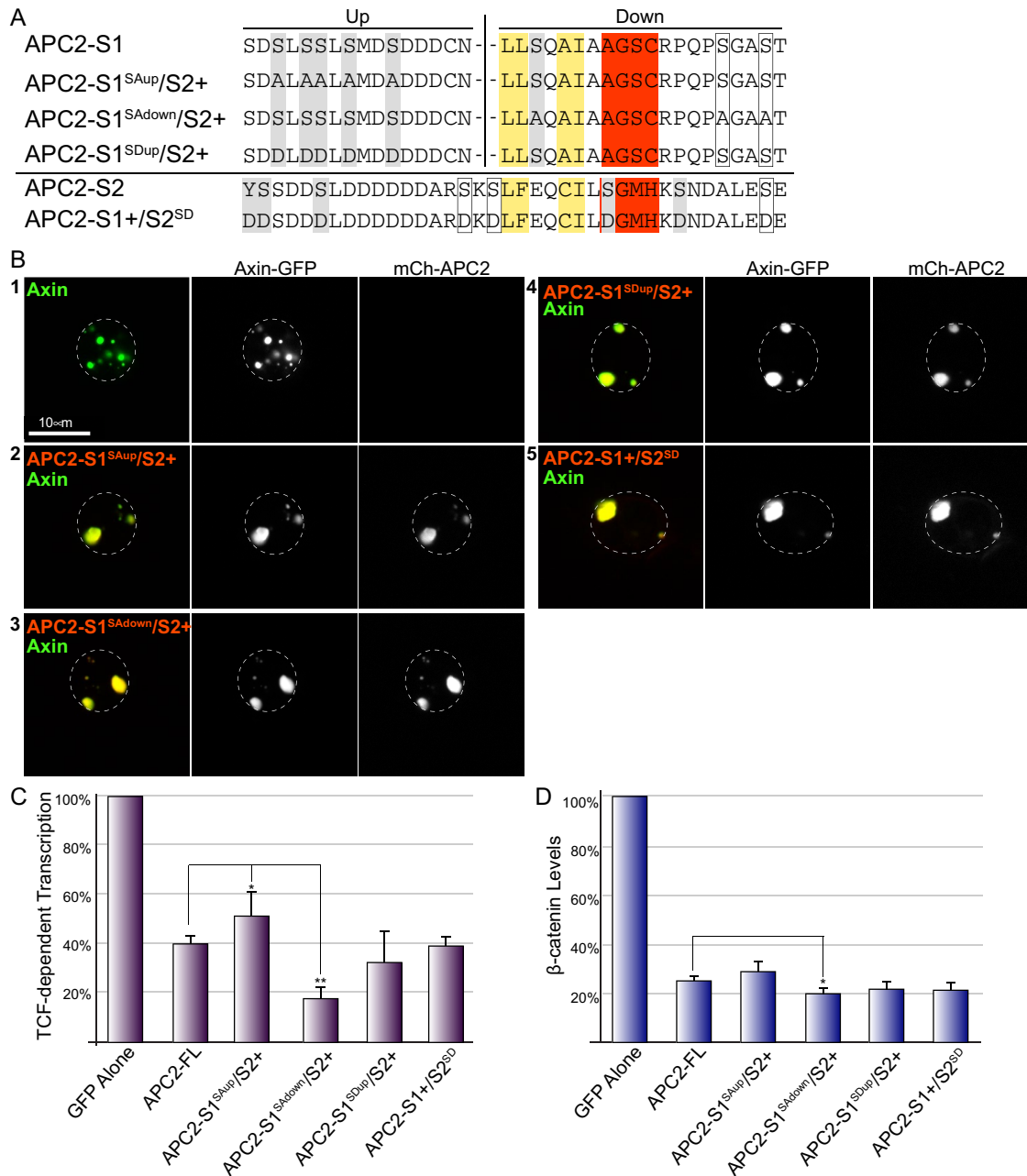
FIGURE 8: Dissection of SAMP phosphorylation. (A) Sequence alignment of the additional phospho-SAMP mutants. SAMP1 was divided into "up" (more N-terminal) and "down" (more C-terminal), and these subsets of phosphoresidues were altered as shown. Residues in gray are predicted phosphorylation sites based on mass spectrometry evidence, and white boxes are predicted phosphorylation sites (based on computational predictions as previously stated. (B) Axin-GFP forms cytoplasmic puncta (1), and Axin-GFP+mCh-APC2-FL colocalizes in the puncta in S2 cells (2). All of the mutant forms of APC2 localized with Axin-GFP in puncta like APC2-FL (see Figure 2). Dotted lines indicate cell boundaries. Scale bar, 10 μm. (C) In SW480 cells, APC2-FL suppresses high levels of target gene activity. Whereas the APC2-S1^{SAup}/S2+ moderately suppressed the elevated levels of Wnt target gene activity, APC2-S1^{SAdown}/S2+ performed better than the FL control. Both phosphomimetic mutants APC2-S1^{SDup}/S2+ and APC2-S1+/S2^{SD} looked similar to the full-length control. (D) Expression of APC2-FL was sufficient to suppress the elevated levels of β-catenin in SW480 cells, and all of the mutants displayed comparable activity to the FL control. APC2-S1^{SAdown}/S2+ performed better than the FL control, similar to the TOPFlash experiments. Error bars represent SD. *$p \leq 0.05$, **$p \leq 0.01$.

Axin-binding domain of APC, and discovered that the two SAMP repeats of APC2 have distinct roles in the destructosome (Figure 9A). In addition, we showed for the first time that the potential phosphorylation of SAMP repeats promotes APC's destructosome function (Figure 9A).

Functional diversity among SAMP repeats

All APC proteins contain an N-terminal region composed of the structured Armadillo repeats followed by variable numbers of β-catenin–binding sites (20Rs) and Axin-binding sites (SAMP motifs) in the C-terminus. These domains (20Rs and SAMPs) show a high

| A	Axin Interaction (Y2H)	Colocalization w/ Axin puncta		Transcriptional Repression (SW480)	Rescue APC2 *null* (APC1+)	Rescue *APC2APC1 null*	Dominant Negative Activity
		S2	SW480				
APC2-FL	++++	Yes	Yes	+++	++++	++++	NA
APC2-S1-/S2+	+++	Yes	Yes	+	++++	+	No
APC2-S1^{SA}/S2+	ND	Yes (Cortical Axin)	Yes	++	++++	-	No
APC2-S1^{SAup}/S2+	ND	Yes	Yes	+++	ND	ND	ND
APC2-S1^{SAdown}/S2+	ND	Yes	Yes	+++	ND	ND	ND
APC2-S1^{SDup}/S2+	ND	Yes	Yes	+++	ND	ND	ND
APC2-S1+/S2-	+	Yes (Cortical Axin)	Yes	+++	++++	-	Yes
APC2-S1+/S2^{SA}	ND	Yes	Yes	+++	++++	++	No
APC2-S1+/S2^{SD}	ND	Yes	Yes	+++	ND	ND	ND
APC2-S1-/S2-	-	No	No	+	+	-	Yes
APC2-S1^{SA}/S2^{SA}	ND	Yes (Cortical Axin)	Yes	++	+++	+	No
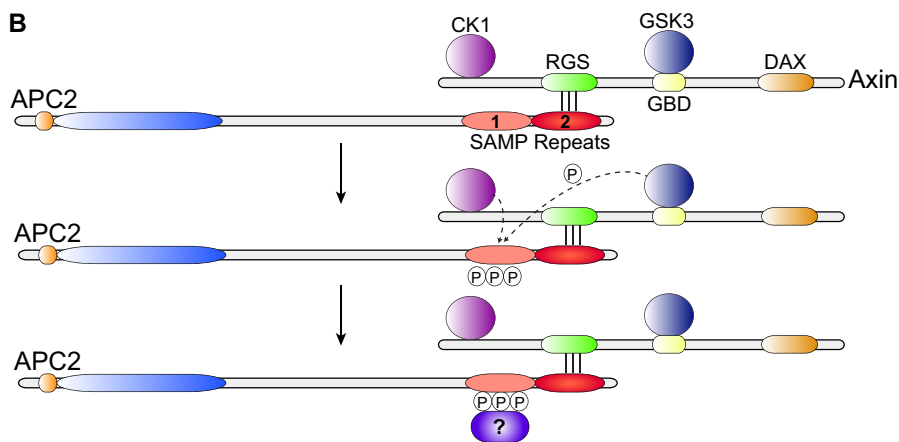
FIGURE 9: Summary and model. (A) Summary of the activities of APC2's two SAMP repeats. (B) Model for a mechanism of cooperation between SAMP1 and SAMP2. The stronger Axin binder, SAMP2, binds to the RGS domain of Axin. This brings GSK3 and CK1 in proximity to APC2, promoting the phosphorylation in SAMP1. Phosphorylated SAMP1 participates in destructosome by increasing the affinity of APC2 and Axin or by recruiting an unknown protein.

invertebrate APC/APC2s (Supplemental Figure S1A). In addition, there is little sequence conservation immediately surrounding the Axin-binding motif among various SAMPs even within the same APC protein. The exception to this rule is high conservation of the upstream phosphoresidues (S-X-X-S-S-L-S-X-L-S followed by downstream acidic residues) between vertebrate and invertebrate SAMP3s (*Drosophila* APC2-SAMP1, *Ciona* APC-SAMP2, and leech APC-SAMP2; Supplemental Figure S1B). Taken together, these observations suggested that the SAMP repeats might be functionally diverse rather than redundant.

Our data strongly support the hypothesis that SAMP repeats are functionally distinct, with differences in Axin-binding ability, effect on Axin localization, importance of phosphorylation, and destructosome function. SAMP1 weakly binds to Axin, yet SAMP1 is sufficient for destructosome function in SW480 cells and, in the presence of APC1, is sufficient in the *Drosophila* embryo as well (Figure 9A). In the absence of all other APC in the embryo, however, SAMP1 is not sufficient, providing no rescue and instead producing a strong dominant-negative effect and enhancement of the double-null phenotype. We predict that SAMP1 alone is sufficient in the presence of APC1 because APC1 and the mutant APC2 bind through the APC self-association domain (Kunttas-Tatli *et al.*, 2014), allowing the mutant to carry out some of APC2's destruction functions via complementation in-*trans*. We previously observed this phenomenon with mutants in the 20Rs (Kunttas-Tatli *et al.*, 2012). Although these results indicate that SAMP2 is necessary for destruction function in the *Drosophila* embryo, it is not sufficient. Deletion of SAMP1 alone produces a protein that retains some function in the absence of all other APC in the embryo (Figure 9A). Surprisingly, APC2 with SAMP2 alone performed very poorly in SW480 cells, indistinguishably from the deletion of both SAMPs. Some of the inconsistencies between the functional significance of various mutants in SW480 cells and flies were observed before when studying APC2. For example, work in human cells showed that overexpression of internal fragments of human APC (containing at least three 20Rs) rescues β-catenin destruction and TOPFlash values (Rubinfeld *et al.*, 1997; Roberts *et al.*, 2011; Li *et al.*, 2012). However, overexpression of analogous fragments of *Drosophila* APC2 in SW480 cells does not rescue either β-catenin destruction or TOPFlash (D.M.R., unpublished results). It is likely that at least some of these inconsistencies are due to the fact that SW480 cells are not null for APC proteins but instead express truncated APC and full-length APC2 (APC-L; Schneikert *et al.*, 2013). Thus, although SW480 cells are useful in testing many aspects of APC biology, it is still a somewhat artificial system and might not represent true in vivo conditions.

Taken together, our results strongly suggest that the two SAMP repeats are not functionally equivalent, and the difference cannot be explained by differences in Axin binding alone. If this were the case, SAMP2 alone would always perform better than SAMP1 alone.

degree of flexibility both in number and organization across various species. The C-terminal region of APC is predicted to be intrinsically disordered, and it has been proposed that this disorder is critical to coordinate APC's various protein interactions (Minde *et al.*, 2011). However, structural disorder likely also provides a mechanism for duplication and evolution of protein-binding motifs without disrupting protein function. This in turn yields possibilities for alternative domain–domain packing and can even lead to functional differences between paralogues. In fact, 20R2 likely provides an example of divergent evolution in APC proteins: it is highly conserved across all APC proteins and matches the consensus 20R sequence, yet 20R2 does not bind β-catenin (Liu *et al.*, 2006). 20R2 is required for APC function in both flies and humans, suggesting that it has acquired a novel function through evolution (Roberts *et al.*, 2011; Schneikert *et al.*, 2014).

The SAMP repeats may provide another example of divergent evolution of protein-binding motifs in APC. APC-SAMP repeats are evolutionarily conserved among all bilaterians, mediating a key interaction between APC and Axin in the destructosome. Given the importance of this interaction in preventing Wnt target gene activation in the absence of a ligand, it is not surprising to find substantial homology of SAMP motifs between divergent species ranging from insects to humans (Supplemental Figure S1A). The number of Axin-binding motifs and their sequence is largely invariant among vertebrate APCs, whereas it is more variable in vertebrate APC2s and all

Moreover, prior work investigating an Axin mutant lacking the RGS domain revealed that the RGS domain is not critical for proper destructosome function in the fly embryo (Peterson-Nedry et al., 2008). This suggests that an RGS/SAMP interaction is not absolutely essential, and SAMP-independent mechanisms for APC/Axin association have been reported, identifying 20R2 and the Arm repeats as putative Axin-binding sites (Roberts et al., 2011; Schneikert et al., 2014; Pronobis et al., 2015). Collectively these findings suggest the SAMP motifs do more than simply bind Axin for destructosome function.

SAMP phosphorylation is necessary for APC2's destructosome function

Our work has revealed that phosphorylation may contribute to the functional differences between SAMP1 and SAMP2. Phosphorylation of the conserved sites within SAMP1 is required for APC2's destruction function, as this mutant lacks all function in the APC double-null embryos. Surprisingly, loss of SAMP1 phosphorylation has a stronger effect on APC2 function than deletion of the repeat (Figure 9A). In addition, our preliminary dissection of putative phosphorylation sites within SAMP1 suggests that the sites upstream of the Axin-binding residues that are 100% conserved with human APC-SAMP3 play a more significant role in destruction function. In contrast, phosphorylation of SAMP2 appears to have only modest effects on APC2 function and is dispensable in both SW480 cells and in embryos expressing APC1. Of interest, blocking SAMP2 phosphorylation in the SAMP1-SA mutant suppresses the APC2-S1^{SA}/S2+ phenotype; APC2-S1^{SA}/S2+ has a stronger loss-of-function phenotype than APC2-S1^{SA}/S2^{SA} (Figures 7A and 9A), suggesting a regulatory relationship between the SAMP repeats.

Surprisingly, the phosphorylation state of SAMP1 also affected the localization of Axin in S2 cells: loss of phosphorylation of SAMP1 alone or both SAMP1 and 2, but not the deletion of SAMP1, resulted in APC2–Axin colocalization in destructosome-like puncta, but the colocalization was frequently at the cell cortex (Figure 9A). Deleting of SAMP2 but leaving SAMP1 intact had the same effect on Axin (Figure 9A). We found these results perplexing, but APC-mediated recruitment of Axin to the cytoskeleton was observed previously (Schneikert et al., 2014), which can be explained via multiple scenarios. First, without Axin, wild-type APC2 is strongly enriched at the cell cortex in S2 cells, as it is in Drosophila tissues (Zhou et al., 2011). We previously showed that APC2 cortical localization is dependent on the N-terminal ASAD (Kunttas-Tatli et al., 2014) and the C-terminal 30 amino acids (C30; Zhou et al., 2011), both of which are present in all of the mutant forms of APC2 expressed in S2 cells. In the presence of sufficient Axin, APC2 is driven into cytoplasmic puncta and completely lost from the cortex (Figure 2B). This suggests that with wild-type proteins, APC2 binds to Axin with a higher affinity than it does to the cortex. If this is the case, one possible explanation for these results is that the mutations increase the affinity of APC2 for its cortical partner(s), such that APC2 remains at the cortex and recruits Axin. Another potential scenario might be that the deletion or disruption of phosphorylation might modify the structure of the APC:Axin complex in such a way that it alters the ability of Axin to polymerize via its DAX domain.

Mechanistic insight into the contribution of the SAMPs to destructosome activity

Evolution of the SAMP motifs may provide insight into their divergent mechanistic functions. Although all vertebrates have two paralogous APC proteins (APC and APC2), the presence of APC2 is fairly restricted in insects, suggesting that this gene duplication occurred

independently and more recently. Therefore the ancestral APC protein likely looked more like APC1, containing a number of 15Rs, 20Rs, and SAMP motifs. Whereas APC1 proteins have retained these features (and perhaps even gained some), APC2 proteins have likely lost some features after their respective gene duplication events. For example, human APC2 lost all 15Rs, whereas Drosophila APC2 lost some SAMP motifs, as well as the C-terminal basic domain and EB1 binding domain (Figure 1A).

Despite the variable number and positioning of the SAMP motifs in APC proteins, sequence conservation of the SLSSLS signature unambiguously reveals that SAMP1 in Drosophila APC2 is analogous to SAMP3 in both human and Drosophila APC1. Of interest, in human APC1, the S-X-X-S-S-L-S-X-L-S signature is part of the adjacent 20R7, which biochemical studies suggest has moderate binding affinity for β-catenin (Liu et al., 2006; Supplemental Figure S1D). 20R7 has been lost in Drosophila APC2, but it appears likely that dAPC2 SAMP1 originated through a duplication event that retained part of this β-catenin binding 20R. 20Rs are characterized by an N-terminal extended region and a downstream phosphoregion, with the S-X-X-S-S-L-S-X-L-S signature constituting the phosphoregion (Supplemental Figure S1D). The extended region is believed to mediate interactions with β-catenin via two critical salt bridges (charged buttons), with CK1 and GSK3 phosphorylation of the phosphoregion increasing the affinity of APC for β-catenin by 300- to 500- fold (Xing et al., 2004). One exciting possibility is therefore that SAMP1 may retain partial binding to β-catenin. To get a better understanding of the evolution and potential role of this hybrid domain, we compared sequence alignments of human APC-20R7SAMP3 with various invertebrates (Supplemental Figure S1, D and E). The partial conservation of the key extended region residues upstream of the S-X-X-S-S-L-S-X-L-S signature in some invertebrates suggests that they likely retain the ability to bind to β-catenin through the hybrid SAMP1 domain, whereas other invertebrates, such as Capitella teleta and Drosophila, have lost this activity due to the loss of critical residues in the extended region (Supplemental Figure S1D). Consistent with this prediction, a mutant form of Drosophila APC2 lacking both 15Rs and 20Rs but retaining this cryptic domain does not interact with β-catenin in Y2H experiments (Yamulla et al., 2014). Alternatively, the S-X-X-S-S-L-S-X-L-S signature is found in other proteins (Marin et al., 2003) and may simply represent a CK1 phosphosignature that allowed SAMP1 to acquire a new and important function.

Regardless of whether SAMP1 retains partial ability to bind β-catenin, the fact that both SAMP repeats are necessary for destructosome function in the fly suggests that they cooperate to achieve β-catenin degradation. We propose a model for how this may occur (Figure 9B). Because of its stronger binding, SAMP2 primarily mediates the Axin interaction. Recruiting APC2 into the Axin complex through SAMP2 brings APC in proximity to GSK3 and CK1 bound to Axin, resulting in phosphorylation of those target sites in SAMP1. Consistent with this model, APC phosphorylation is enhanced by APC–Axin binding (Rubinfeld et al., 2001). Once phosphorylated, how does SAMP1 participate in destructosome function? As mentioned earlier, similar phosphosignatures occur in the APC 20Rs, where they have been implicated in enhanced β-catenin binding (Ha et al., 2004; Liu et al., 2006). Similarly, phosphorylation of 20R2, the essential 20R with no β-catenin–binding activity (Liu et al., 2006), likely mediates an interaction with an unknown APC partner, such as Axin (Schneikert et al., 2014) Thus we speculate that phosphorylation of SAMP1 could likewise mediate an interaction between APC and another destructosome component that is necessary for maximal destruction activity. It is plausible that this partner could be β-catenin or Axin itself. The majority of putative phosphorylation sites are outside

the core α-helix of the SAMP motif as defined in the crystal structure (Spink *et al.*, 2000), suggesting that if such an interaction with Axin occurs, it may be through a "clamp-down" mechanism.

In conclusion, our results suggest that the role of APC's SAMP repeats in the destructosome is far from simple. The two SAMP repeats in *Drosophila* APC2 are functionally distinct, appear to act cooperatively in some contexts, and have more complex regulatory relationships in others. Rather than being a simple bridge connecting the Axin–kinase complex to β-catenin, APC proteins appear to have a much more complex, multifaceted mechanistic role in the destructosome. This work adds a significant new twist to what we believed was an important but rather simple binding motif. Future studies are needed to test our model and reveal precisely how the individual SAMP repeats and their interplay drives β-catenin destruction.

MATERIALS AND METHODS
Constructs and molecular biology
For generating the SAMP-deletion mutants, we used standard PCR-based site-directed mutagenesis to delete SAMP1, SAMP2, or both. For generating the phosphodeficient SAMP mutants, we used the GENEWIZ Custom Gene Synthesis service (GENEWIZ, Plainfield, NJ) to synthesize a region that spanned the SAMP repeats, which then was cloned into the full-length APC2 backbone. We subcloned the desired APC2 constructs into the *Eco*RI site in pRmHa-3-mCherry (metallothionein promoter vector) for S2 cells and pCS2(+)-mCherry (CMV promoter vector) for SW480 cells. For GFP-tagged APC2 constructs in SW480 cells, the APC2 coding region was shuttled to the pDONR Gateway entry vector via PCR. Entry vectors were fully sequenced and then cloned into a modified pECFP-N1 vector (Clontech, Mountain View, CA) containing an N-terminal GFP tag and a Gateway cassette (Roberts *et al.*, 2011). Modified pCaSpeR-2 containing the native APC2 promoter and GFP was used for the expression in *Drosophila* (McCartney *et al.*, 2006). The mutant constructs were confirmed by sequencing. APC2-FL: 1–1067; APC2-S1–/S2+: 1–930 plus 992–1067; APC2-S1+/S2–: 1–992 plus 1037–1067; APC2-S1–/S2–: 1–930 plus 1037–1067; APC2-S1^{SA}/S2+: S933A, S935A, S936A, S938A, S941A, S949A, S962A, S965A; APC2-S1+/S2^{SA}: Y997A, S998A, S102A, S112A, S114A, S122A, S127A, S133A; APC2-S1^{SA}/S2^{SA}: S933A, S935A, S936A, S938A, S941A, S949A, S962A, S965A, Y997A, S998A, S102A, S112A, S114A, S122A, S127A, S133A; APC2-S1^{SAup}/S2+: S933A, S935A, S936A, S938A, S941A; APC2-S1^{SAdown}/S2+: S949A, S962A, S965A; APC2-S1^{SDup}/S2+: S933D, S935D, S936D, S938D, S941D; and APC2-S1+/S2^{SD}: Y997D, S998D, S102D, S112D, S114D, S122D, S127D, S133D.

Yeast two-hybrid analysis
The Matchmaker System (Clontech) was used to perform the Y2H analysis as described previously (Kunttas-Tatli *et al.*, 2014). APC2 constructs containing the various C-terminal domains and Axin-RGS (see Figure 1) were cloned into the pCR8/GW/TOPO vector (Life Technologies, Grand Island, NY) and LR reactions performed to clone inserts into pGBKT7-W and pGADT7-W. β-Galactosidase assays performed using the Yeast β-galactosidase Assay Kit (Thermo Scientific, Rockford, IL), and the activity was calculated from activity = $(1000 \times OD_{420})/(TV \times OD_{660})$, where *T* is the duration of the reaction in minutes and *V* is the volume of the reaction in milliliters. Construct boundaries are as follows: APC2-C-term-SAMP1 (890–966), APC2-C-term-SAMP2 (970–1037), and APC2-C-term-SAMP (890–1037).

S2 cell culture and transfection experiments
Drosophila S2 cells were cultured in Schneider's medium (Lonza, Basel, Switzerland) with 10% heat-inactivated fetal bovine serum (FBS)

and 1× penicillin–streptomycin (Pen/Strep) at 25°C. pRmHa-3-mCherry (metallothionein promoter vector) constructs were transfected into S2 cells following the Effectene (Qiagen, Valencia, CA) protocol at a cell density of $(2–3) \times 10^5$ cells in six-well plates. Expression of constructs was induced 24 h posttransfection with $CuSO_4$ (40 mM final concentration) and imaged 14–16 h postinduction. BD FacsVantage Diva option (laser 488) was used to sort the highest GFP (top 30%)–expressing S2 cells 16–18 h postinduction for a subset of the experiments. Cells were sorted into glass-bottom dishes (MatTek) with 1× phosphate-buffered saline (PBS), and images of live cells were taken immediately for visualization of puncta morphology.

SW480 cell culture, transfections, and immunofluorescence
Human colon cancer SW480 cells were cultured in DMEM with high glucose supplemented with 10% heat-inactivated FBS and 1× Pen/Strep/Glutamine at 37°C and 5% CO_2. For transfections, SW480 cells were plated at a density of 2.5×10^5 cells in six-well plates, grown overnight, and transfected with TurboFect according to the manufacturer's protocol (Thermo Fisher). Cells were processed 24 h posttransfection. For immunofluorescence, cells were fixed and stained as previously described (Roberts *et al.*, 2011).

TOP/FOP luciferase reporter assay
Luciferase assays were performed using the Dual Glo Luciferase System (Promega, Madison, WI) according to the manufacturer's protocol and also as previously described (Kunttas-Tatli *et al.*, 2014). Luciferase signal was normalized to *Renilla* activity and overall values normalized to the mCherry only control. The TOP/FOPFlash luciferase constructs and the pRL *Renilla* transfection control were provided by Hans Clevers (Hubrecht Institute, Utrecht, Netherlands). All samples were measured in triplicate per experiment, and two or three independent experiments were performed. None of the constructs displayed significant FOPFlash activity (unpublished data). Student's *t* test was used to determine the statistical significance of the averages of different mutants.

Quantifying β-catenin protein levels in transfected SW480 cells
The β-catenin protein levels in transfected SW480 cells were measured using flow cytometry. Cells were trypsinized, fixed in 10% formaldehyde/1× PBS for 20 min, and then permeabilized with 1× Perm/Wash reagent (BD Biosciences, San Jose, CA). Antibody staining was in 1× Perm/Wash with mouse anti–β-catenin (1:1000; BD Transduction) followed by goat anti-mouse Alexa 647 (1:1000; Life Technologies). Stained cells were analyzed on an Accuri C6 Flow Cytometer, and the mean fluorescence intensity of GFP-positive cells was determined. At least 10,000 total cells were analyzed per sample, and four independent experiments were performed. The mean fluorescence intensity of transfected cells was first normalized to that of untransfected cells to account for staining variability between samples. These values were then normalized to the GFP-only control. Student's *t* test was used to determine the statistical significance of the averages of different mutants.

Fly genetics, hatch rate, and cuticle analysis
Transgenic flies expressing *P[endoP-EGFP-APC2-FL]* (Zhou *et al.*, 2011), as well as the rest of the SAMP mutants (*P[endoPEGFP-APC2-S1-/S2+]*, *P[endoPEGFP-APC2-S1+/S2–]*, *P[endoPEGFP-APC2-S1-/S2–]*, *P[endoPEGFP-APC2-S1^{SA}/S2+]*, *P[endoPEGFP-APC2-S1+/S2^{SA}]*, *P[endoPEGFP-APC2-S1^{SA}/S2^{SA}]*) were generated using P-element–mediated germline transformation (Model System Genomics; Duke University, Durham, NC). Transgenes on the

second chromosome for each construct were crossed into the $APC2^{g10}$ (APC2 null) or $APC2^{g10}APC1^{Q8}$ (double null) backgrounds using standard methods (Supplemental Figure S4C). Hatch rate analysis was performed and embryonic cuticles were prepared as previously described (Wieschaus and Nusslein-Volhard, 1998). Scoring criteria for the cuticle phenotype were previously described (McCartney et al., 2006). Cuticle images were taken at the same magnification with dark-field illumination and a 20× objective.

Immunohistochemistry and immunoblotting in the *Drosophila* embryo

Embryos were collected for 4-6 h at room temperature and fixed and stained as previously described (McCartney et al., 1999). Anti-Engrailed (En; mouse, 4D9, 1:50), and anti-Armadillo (Arm; mouse, N27A1, 1:250) were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa (Iowa City, IA). Anti-GFP (1:5000; Abcam, Cambridge, MA) was preabsorbed against w^{1118} embryos before use for immunohistochemistry. Secondary antibodies were conjugated with various Alexa dyes (1:1000; Invitrogen, Carlsbad, CA). Equal volume of wild-type and transgenic embryos (0–6 h at 27°C) were lysed in lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5, 115 mM KAc, 2.5 mM Mg(Ac)2, 0.5% Nonidet P40 substitute [Sigma-Aldrich, St. Louis, MO], 0.5 mM EDTA, 0.5 mM ethylene glycol tetraacetic acid, 1× Complete protease inhibitor cocktail [Roche, Basel, Switzerland], and 1× phosphatase inhibitor cocktail 2 [Sigma-Aldrich]). Embryo lysates were then analyzed by 6% acrylamide SDS–PAGE, transferred to nitrocellulose membrane, and immunoblotted with rat anti-APC2 (1:1000) antibody. Ponceau stain (Sigma-Aldrich) was used to visualize equal loading.

Imaging and image analysis

A Zeiss (Thornwood, NY) Axiovert 200M spinning disk confocal microscope with a Yokagawa scan head (Solamere Technology Group, Salt Lake City, UT) and a QICAM-IR camera (Qimaging) was used to acquire the images of both cells and embryos using the QED InVivo software. For images of whole embryos stained for Arm and En, multiple fields were captured with a 25× objective and merged with Photoshop (Adobe) postimage processing to generate whole-embryo images.

Sequence alignments

APC sequences with accession codes used in the alignments are as follows. Vertebrates: *Homo sapiens* (human): *APC* AAA03586.1, APC2 NP005874; *Mus musculus* (mouse): APC XP196187, APC2 NP035919.2; *Gallus gallus* (chicken): APC XP004949340, APC2 XP426919; *Anolis carolinensis* (lizard): APC XP003223070 XP008122667, APC2 XP008122667.1; *Xenopus tropicalis* (frog): APC XP002936457.1, APC2 XP002941166; and *Danio rerio* (zebrafish): APC NP001137312.1, APC2 XP_009304120. Invertebrates: *Ciona intestinalis* (sea squirt): APC XP002124987.2; *Saccoglossus kowalevskii* (acorn worm): XP002738523.1; *Strongylocentrotus purpuratus* (sea urchin): (XP783363.3); *Lottia gigantean* (snail): APC ESO95067.1; *C. teleta* (polychaete worm): APC ELU12449.1; *Helobdella robusta* (leech): XP009018419.1; *Limulus polyphemus* (horseshoe crab) XP013781878.1; *Octopus bimaculoides* (octopus) gb-KOF62912.1; *Drosophila melanogaster* (fruit fly): APC1 AAB41404.1, APC2 AAF56249.1; *Anopheles gambiae* (mosquito): AGAP000048; *Tribolium castaneum* (beetle): XP_008198134; *Bombus terrestris* (bee): APC XP003403146.1; and *Nasonia vitripennis* (wasp): APC XP001602839.2. ClustalW was used for generating sequence alignments.

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