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Region-specific proteolysis differentially modulates type 2 and type 3 inositol 1,4,5-trisphosphate receptor activity in models of acute pancreatitis

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Running title: *Proteolytic regulation of IP_3R2 and IP_3R3*

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Abstract

Fine tuning of the activity of Inositol 1,4,5-trisphosphate receptors (IP_3R) by a diverse array of regulatory inputs results in intracellular Ca^{2+} signals with distinct characteristics. These events allow the activation of specific downstream effectors. We previously reported that region-specific proteolysis represents a novel regulatory event for type 1 IP_3R (R1). Specifically, caspase fragmented R1 display a marked increase in single channel open probability. More importantly, the distinct characteristics of the Ca^{2+} signals elicited *via* fragmented R1 can activate alternate downstream effectors. In this report, we expand these studies to investigate if all IP_3R subtypes are regulated by proteolysis. We now show that type 2 and type 3 IP_3R (R2 and R3, respectively) are proteolytically cleaved in rodent models of acute pancreatitis. Surprisingly, fragmented IP_3R retained tetrameric architecture, remained embedded in ER membranes and were not functionally disabled. Proteolysis was associated with a marked attenuation of the frequency of Ca^{2+} signals in pancreatic lobules. Consistent with these data, expression of cDNAs encoding complementary R2 and R3 peptides mimicking fragmented receptors at particular sites, resulted in a significant decrease in the frequency of agonist-stimulated Ca^{2+} oscillations. Further, proteolysis of R2 resulted in a marked decrease in single channel open probability. Taken together, proteolytic fragmentation modulates R2 and R3 activity in a region-specific manner and this event may contribute to the altered Ca^{2+} signals in pancreatic acinar cells during acute pancreatitis.

Introduction

Inositol 1,4,5-trisphosphate receptors (IP_3R) are ubiquitous, intracellular Ca^{2+} release channels expressed predominantly in endoplasmic reticulum (ER) membranes (1-4). IP_3R can encode Ca^{2+} changes with distinct spatial and temporal characteristics and these signals subsequently play essential roles in controlling a plethora of biological processes (1,2,5-7). The versatility of these signals is a consequence in large part, of the regulation of IP_3R activity at multiple levels. First, there are three isoforms of IP_3R, termed the R1, R2 and R3, encoded by three different genes (1). IP_3R can either form homo- or hetero-tetrameric channels. The composition of the assembled tetramer either dictates or contributes to the channel activity (8). Further, binding of numerous molecules can regulate IP_3R properties including the pharmacological and biophysical characteristics, together with receptor localization (9,10). Another level of modulation occurs as a function of post-translational modification including phosphorylation and ubiquitination events, which can either alter channel activity or determine channel abundance (2,11). Recently, our lab has demonstrated that region-specific proteolytic fragmentation dramatically alters R1 activity and thereby allows the receptor to potentially activate

alternative downstream effectors (12). However, whether this is a regulatory event specific to R1, or a general form of regulation relevant to all isoforms of IP_3R, requires further investigation. Moreover, whether proteolysis of R2 and R3 has similar effects on the biophysical properties of all subtypes, remains to be established.

R2 and R3 play pivotal roles in exocrine secretory systems (13-15). For example, R2- and R3-mediated Ca^{2+} signals play a functionally dominant role in the exocytosis of stored zymogens from the pancreas, which along with the production of a NaCl-rich fluid, is the primary function of acinar cells. The central role of R2/R3 is most strickingly demonstrated by the observation that R2 and R3 compound knockout mice failed to thrive after weaning due to a severe disruption of exocrine function. Indeed, Ca^{2+} transients and amylase secretion in response to secretagogue-stimulation were reported to be completely abolished in pancreatic acinar cells isolated from R2 and R3 double-knockouts (13).

R2 and R3 share the same general primary structure as R1, and therefore are predicted to be subject to cleavage by proteases, at similar solvent exposed sites (16). An early report suggested that R2 and R3 are substrates of caspase and calpain, respectively (17). In addition, our laboratory reported that in a model of acute pancreatitis that R3 were cleaved into low molecular weight receptor fragments and inhibition of proteasome activity failed to completely prevent receptor fragmentation (18). These observations imply that R3 were cleaved intracellularly, by inappropriately activated digestive enzymes such as trypsin and chymotrypsin within pancreatic acinar cells during acute pancreatitis (16,19,20). Of note, both proteases are reported to cleave IP_3R *in vitro (16,19-21)*. This evidence suggests that models of acute pancreatitis may represent an ideal experimental platform to investigate the *in vivo* consequences of R2 and R3 fragmentation and subsequent effects on agonist-stimulated $[Ca^{2+}]_i$ signals.

In the current study, utilizing both *in vivo* and *in vitro* rodent models of acute pancreatitis, we demonstrate that R2 and R3 are substrates for proteases. Moreover, proteolysis results in channels that retain fundamental tetrameric architecture and remain in ER membranes, but exhibit dramatically altered channel activity. Specifically, in both pancreatitis models and in expression systems mimicking fragmentation, agonst-stimulated Ca^{2+} oscillations are attenuated and channel activity is reduced. This study therefore indicates that proteolysis is a novel mechanism for regulating R2 and R3 activity. Furthermore, while proteolysis modulates the activity of all IP_3R isoforms, intriguigingly, it does so in a subtype and fragmentation pattern specific manner.

Results

Generation of fragmented R2 and R3 in an *in vivo* acute pancreatitis model.

To model acute pancreatitis, mice received three consecutive intraperitoneal (i.p.) injections of a supramaximal concentration of secretagogue analogue, caerulein (50 µg/kg/injection) or saline as a control. This approach represents a well-established, reversible and relatively non-invasive mouse model of the disease (22,23). Previous studies have reported that while isolated pancreatic acinar cells (PAC) from healthy mice displayed robust Ca^{2+} oscillations in response to low concentrations of secretagogues, those from mice treated with this protocol evoked a single Ca^{2+} transient or repetitive Ca^{2+} transients with a significantly lower frequency (24). We first performed $[Ca^{2+}]_i$ measurements in PAC within excised pancreatic lobules, as previously described (15). This paradigim allows the analysis of Ca^{2+} signaling dynamics in PAC in a more native enviroment and without the time consuming process of enzymatic digestion of pancreatic tissue needed to produce isolated acinar cells. Upon carbachol (CCh) (300 nM) stimulation, PAC from control mice evoked robust Ca^{2+} oscillations with each spike returning close to the basal Ca^{2+} level between each elevation. The oscillatory Ca^{2+} signals persisted beyond 30 min of the recording (Figure 1A and D). Consistent with previous studies (24,25), PAC from acute pancreatitis mice elicited Ca^{2+} signals with a significantly lower frequency (Figure. 1B and D). Moreover, a higher concentration of CCh (500 nM) failed to rescue the ability to induce strong Ca^{2+} oscillations in PAC from caerulein treated mice (Figure 1C and D). This strongly indicates that the alteration of Ca^{2+} signals during acute pancreatitis is not likely due to

desensitization to the stimuli, but may result from modifications of the constituent components promoting Ca^{2+} signaling.

It is well accepted that Ca^{2+} oscillations in pancreatic acinar cells are absolutely dependent on the activity of IP_3R in the initial phase and subsequently on both IP_3R and Orai-based channels during sustained stimulation (13,26). We therefore postulated that the remarkable reduction in oscillatory Ca^{2+} signals observed, (Figure 1B, C and D) resulted from altered regulation of IP_3R-induced Ca^{2+} release. While all three isoforms of IP_3R are expressed in the pancreas, R2 and R3 are most abundant and functionally dominant (27,28). Therefore, we performed western blot assays to investigate if R2 and R3 are modified in this model of acute pancreatitis. Consistent with our previous report, secretagogue-induced hyperstimulation significantly downregulated both the full-length R2 and R3 (Figure 2A, B, F, G) (18). In addition, we also observed the generation of both fragmented R2 and R3 receptors in mice treated with caerulein (Figure 2A, C, D, F, H, I). Given the strong evidence that digestive enzymes such as trypsin and chymotrypsin, are prematurely activated as an early event in PAC in models of acute pancreatitis (29-32), we speculated that IP_3R were fragmented by intracellularly active digestive enzymes. Previous studies demonstrated that exposure of IP_3R to low concentrations of trypsin and chymotrypsin *in vitro* resulted in five receptor fragments (16,21,33). These results have been interpreted to indicate that IP_3R, structurely consists of five compact globular domains (IP_3R fragment I, II, III, IV and V) interconnected with four solvent exposed linker regions. To estimate the relative sizes of receptor fragments and the cleavage sites on the IP_3R in PAC from mice treated with caerulein, we constructed a library of cDNA encoding R2 or R3 complementary receptor fragments based on the previously reported tryptic cleavage sites of the receptors (Figure 3A and D) (16). Each cDNA was then stably transfected into an IP_3R *null* background cell line, DT40-3KO (Figure 3B, C, E, F). Fragmented R2 mimicking cleavage at the tryptic site in the second solvent exposed region was named as R2 I-II+III-V (tryp). According to this nomenclature, we generated R2 I-II+III-V (tryp) and R2 I-III+IV-V (tryp) for R2, and R3 I-II+III-V (tryp), R3 I-III+IV-V (tryp) and R3 I-IV+V (tryp) for R3. In addition, we also generated R2 I-IV+V (calpain) based on the sequence homology between IP_3R1 (R1) and R2 (Figure 3A-F). Notably, receptor fragments in PAC from caerulein-treated mice mainly migrated between 250 and 150 kDa, which were at the similar molecular weights as R2 I-III (tryp), R2 I-IV (calpain), R3 I-III (tryp) and R3 I-IV (tryp), indicating R2 and R3 were preferentially fragmented at the third and fourth solvent exposed domains (Figure 2A, F). Moreover, the N-terminal fragments of cleaved R2 co-immunoprecipitated with the C-terminal fragments (Figure 2E) in the pancreatic sample prepared from mice treated with caerulein, strongly suggesting that IP_3R remained associated after proteolysis during the development of models of acute pancreatitis. Trypsin as one of the essential digestive enzymes is activated during acute pancreatitis, which has been shown to cleave R2 and R3 *in vitro* (20,34). To test the involvement of trypsin activity in receptor fragmentation, we performed the *in vivo* acute pancreatitis model in Trypsinogen 7 knockout mice. These animals are reported to have a 60% reduction in total trypsinogen content, the precursor of trypsin (35). While genetic knockout of trypsinogen 7 had less effect on generation of R2 fragments (Figure 4A and C), the elevation R3 fragments was significantly reduced in this model of acute pancreatitis (Figure 4B and D). Therefore, while these data suggest a role of trypsin in receptor fragmentation, they also indicate that other proteases likely participate in receptor proteolysis.

Generation of fragmented R2 and R3 in an *in vitro* acute pancreatitis model.

To further investigate R2 and R3 receptor fragmentation specifically in PAC, we next exploited a well-established *in vitro* pancreatitis model using isolated rat PAC (36). To mimic features of pancreatitis *in vitro*, isolated rat PAC were incubated with two supramaximal concentrations of CCh for different time periods followed by western blot analysis (37). PAC had a relatively low level of fragmented R2 (Figure 5A) and R3 (Figure 5B) under control conditions, presumably as a result of prematurely activated proteolytic enzymes resulting from the inevitable cell damage occuring during pancreatic acinar cell preparation and manipulation. However, we cannot

exclude the possibility that there is a basal level of fragmented R2 and R3 under physiological conditions. Nevertheless, in agreement with our *in vivo* findings, despite the appearance of low amounts of fragmented R2 and R3 without treatment, supramaximal concentrations of CCh consistently, in a concentration-dependent manner, further increased the levels of fragmented R2 (Figure 5A) and R3 (Figure 5B) accompanied by a concomitant reduction of full length receptor. To demonstrate that fragmentation of IP_3R in PAC was not limited to the hyperstimulation model of acute pancreatitis, PAC were exposed to the bile acid, taurolithocholic acid 3-sulfate disodium salt (TLCS) (38-40). This paradigm is often utilized as a pathologically relevant stimulus to mimic pancreatic duct obstruction and subsequent bile reflux. TLCS exposure consistently resulted in increased fragmentation of R2 and R3 (Figure 5C, D, E). Importantly, the generation of receptor fragments could be reversed by pre-incubation of the cells with cell permeable trypsin inhibitors (Figure 5C, D). Together with Figure 4, these data confirm that trypsin activity, directly or indirectly, is responsible for R2 and R3 fragmentation during the development of acute pancreatitis.

Our previous studies investigating the consequences of R1 fragmentation have demonstrated that R1 fragments remain associated within the ER, despite the loss of peptide continuity and retain the ability to be gated by IP_3 (41). Thus, we next investigated if R2 and R3 similarly retains tetrameric architecture and ER membrane localization after receptor fragmentation. Isolated rat PAC were incubated with supramaximal concentrations of CCh to induce acute pancreatitis *in vitro*. Membrane and cytosolic fractions were then prepared by differential centrifugation. GAPDH was used as a marker for the cytosol fraction. No detectable R2 and R3 signals were observed in the cytosol fraction and both N- and C-terminal fragments of R2 (Figure 6A) and R3 (Figure 6B) were present in the membrane fraction indicating, that like R1, R2 and R3 remained ER associated after proteolytic fragmentation. Native, non-denaturing gel analysis showed that fragmented R2 (Figure 6C) and R3 (Figure 6D) in PAC from rats subjected to the *in vitro* model of acute pancreatitis migrated at the same molecular weight as that of the full-length tetramers. Further, no detectable receptor fragments of lower molecular weights were present in any cell lysates under the pancreatitis conditions. In total, these data, strongly suggest that all R2 and R3 exist as tetramers on the ER membrane after proteolytic fragmentation.

Region-specific fragmentation regulates temporal characteristics of Ca^{2+} signals mediated by R2 and R3.

Given the critical roles of IP_3R in Ca^{2+} signaling in PAC (13), our data thus far, led us to posit that proteolytic fragmentation mediates the alteration of R2 and R3 activities and consequently contributes to the altered Ca^{2+} signals in pancreatic acinar cells during the development of acute pancreatitis. To unambiguously study the function of only fragmented R2 and R3, without the potential confounding impact of contaminating signals from endogenous receptors, we performed single cell Ca^{2+} imaging assays using DT40-3KO cells stably expressing complementary peptides to mimic R2 or R3 fragmented at different sites (Figure 3). We have previously demonstrated that after expression, these fragments are assembled into functional tetrameric IP_3R on the ER membrane and are properly gated by IP_3 (41). Consistent with our previous findings, all R2 and R3 generated from cDNAs encoding complementary fragments were capable of supporting Ca^{2+} signals in response to maximal Protease Activated Receptor 2 (PAR2) activation with trypsin (Figure 7A-H).

Next, we investigated if receptor fragmentation has an impact on the temporal characteristics of IP_3R-mediated Ca^{2+} signals. Anti-IgM crosslinks B-cell receptors on the cell surface of DT40-3KO cells and results in the continuous production of IP_3. Upon anti-IgM stimulation, DT40-3KO cells stably expressing either full-length R2 or R3 elicited robust Ca^{2+} oscillations in an isoform-specific manner (Figure 8A and G) (42-44). When cDNAs encoding complementary polypeptides to mimic receptor fragmentation introduced at the second solvent exposed region were expressed, R2 I-II+III-V (tryp) and R3 I-II+III-V (tryp) were still capable of evoking strong oscillatory Ca^{2+} signals (Figure 8B, E, H, K). However, anti-IgM stimulation of cells expressing constructs mimicking receptor fragmentation in R2

and R3 at solvent exposed sites more toward to the C-terminus resulted in Ca^{2+} oscillations with a significantly lower frequency (Figure 8C, D, E, I, J, K). Further, increasing the concentration of anti-IgM failed to rescue the loss of Ca^{2+} oscillations mediated by the R2 I-III+IV-V (tryp), R2 I-IV+V (calpain) R3 I-III+IV-V (tryp) or R3 I-IV+V (tryp) (Figure 8F and 7L). These data strongly suggest that region-specific receptor fragmentation regulates R2 and R3 activities. These results were consistent with those obtained from the *ex vivo* pancreatic acini Ca^{2+} imaging assays (Figure 1A-D), and provide a possible explanation for the reduction of Ca^{2+} oscillations in PAC isolated from mice subjected to caerulein treatment. Based on these data, it can be envisioned that prematurely activated digestive enzymes cleave R2 and R3 at the third and fourth solvent exposed regions, and, as a consequence, significantly reduce the frequency of oscillatory Ca^{2+} signals of IP_3R in PAC during the development of pancreatitis.

One caveat of studying the functional consequences of receptor fragmentation by using complementary receptor fragments is that fragmented IP_3R are assembled from polypeptides, but not generated *in situ*. In addition, measurements of global $[Ca^{2+}]_i$ are an indirect measurement of channel activity. Therefore, we next performed single channel, patch-clamp recording with the "on-nucleus" configuration to study the potential biophysical alteration of the R2 activity resulting from proteolytic fragmentation. An initial attempt was made to induce fragmentation of R2 with trypsin. However, no channel activity was recorded in the presence of trypsin, even at picomolar levels of enzyme, presumably due to the receptor destruction resulting from failure to control the amount of receptor proteolysis under these conditions. To circumvent this difficulty and fragment the receptor specifically at the fourth solvent exposed region *in situ* in a controlled manner, we constructed a R2 with a Tobacco Etch Virus (TEV) protease cleavage sequence inserted immediately after the tryptic site (Arg-1884) in this region. This construct, termed R2 (TEV), was stably expressed in DT40-3KO cells. TEV protease was capable of specifically cleaving purified R2 (TEV) at the fourth solvent exposed region to result in fragments corresponding to R2 I-IV+V (TEV) (Figure 9C). In contrast, incubation of purified full-length R2 with TEV protease failed to result in any receptor fragment (Figure 9A), confirming the specificity of TEV protease-mediated receptor fragmentation. Patch-clamp recording, in the "on-nucleus" configuration demonstrated no difference in the single channel open probability (Po) or mode of gating between R2 (TEV) and full-length R2 WT in the absence of TEV protease (Figure 9B, D, E, F, G). However, when R2 (TEV) was exposed to TEV, a significant decrease in channel Po, accompanied by a significant increase in interburst interval, but with no change in burst length (Figure 9D, E, F, G) was observed. TEV protease had no effect on full-length R2 (Figure 9B, E, F, G), strongly indicating that the alteration in R2 (TEV) channel biophysical properties resulted from receptor fragmentation. Taken together, these single channel data are consistent with the results of the single cell Ca^{2+} imaging assays (Figure 9) and provide an underlying biophysical mechanism to explain the decrease in oscillatory Ca^{2+} signals in PAC during experimental acute pancreatitis (Figure 1).

Discussion

The current study represents a comprehensive investigation of a novel modification of R2 and R3 and its consequences in PAC in the context of two rodent models of acute pancreatitis. A previous study in which PAC were isolated by enzymatic digestion following induction of experimental pancreatitis reported a significant decrease in the ability of PAC from the experimental pancreatitis animals to display sustained oscillatory Ca^{2+} signals. These data are established as strong evidence that the characteristics of the cytosolic Ca^{2+} signal, important for driving physiological secretion is disrupted in experimental pancreatitis (24). The underlying mechanism for this striking transition in Ca^{2+} signaling was not, however elucidated. In this study, we first confirmed this alteration of Ca^{2+} signals in PAC *in situ* in excised pancreatic lobules prepared from control or from mice subjected to the experimental acute pancreatitis paradigm. This preparation has the significant advantage that the tissue is not exposed to prolonged enzymatic isolation and better reflects the heterogeneity of the impact to different regions of the tissue in the pancreatitis model. Notably, western blot analysis demonstrated that concurrent with a significant reduction in the ability of PAC from experimental pancreatitis animals to support sustained Ca^{2+}

oscillations, these mice showed a marked decrease in the abundance of full-length R2 and R3 with the concomitant appearance of various species of fragments of R2 and R3. These observations, coupled with data that proteolytic cleavage of R1 alters the receptors activity without disabling the channel, led us to hypothesize that during pancreatitis, proteases are prematurely activated resulting in fragmentation of R2 and R3 and in turn altered $[Ca^{2+}]_i$ signals.

To explore this hypothesis, experiments were designed to answer four major questions: 1) Do R2 and R3 retain their tetrameric architectures after proteolytic fragmentation? 2) Do fragmented R2 and R3 remain associated with ER membranes? 3) Do fragmented R2 and R3 retain the ability to be gated by IP_3 binding? 4) Does receptor fragmentation alter the temporal profile of agonist-evoked Ca^{2+} signals? First, non-denaturing, native gel analysis combined with co-immunoprecipitation assays strongly suggested that although peptide continuity was lost, R2 and R3 retain tetrameric structure after receptor fragmentation. This is likely due to significant non-covalent interactions between tightly folded globular domains in the proteolytically cleaved protein. Further, membrane fractionation followed by western blot analysis indicated that all fragments of R2 and R3 were still located on ER membranes. To study the function of fragmented receptors, we generated a library of DT40-3KO cells, stably expressing R2 or R3 assembled from complementary peptides representing one type of fragmented IP_3R. Consistent with our previous findings for R1, all fragmented IP_3R could support Ca^{2+} release in response to IP_3 binding. However, when receptors were fragmented more toward to the C-terminus, the temporal characteristics of Ca^{2+} signals were greatly altered. Indeed, compared to full-length receptors, R2 I-III+IV-V, R2 I-IV+V, R3 I-III+IV-V, and R3 I-IV+V exhibited a significantly lower frequency of Ca^{2+} oscillations. At a biophysical level, this observation was supported mechanistically by single channel patch-clamp data, which revealed that receptor fragmentation dramatically decreased single channel open probability.

The current study and our previous report have thoroughly investigated the functional consequences of IP_3R fragmentation (12,45,46). Early studies showed that exposure of purified R1 to a low concentration of trypsin *in vitro* resulted in five receptor fragments (16,21,33). This result was interpreted as the overall structure of R1 consists of five compact globular domains linked by four solvent exposed regions. Based on the sequence homology among all three isoforms of IP_3R, R2 and R3 were also predicted to have the same overall structure (16,19). It is worth noting that all putative fragmentation sites including those reported in the current study are located in these solvent exposed regions and may be explained by the relatively ease of accessibility of these regions to protease activity (16,21,33,47,48). The coupling domain (amino acid 586-2276) of IP_3R generates interfaces where Ca^{2+}, regulatory proteins, and nucleotides bind to mediate channel activities (1,2,49). The distinct regulatory events among the three isoforms of IP_3R confer subtype specific Ca^{2+} signals. We postulate that some of the regulatory events rely on peptide continuity for an appropriate communication between regulatory inputs and the channel domain. As a result, disruption of receptor continuity by proteases in specific regions of the coupling domain would potentially impact such communication, and subsequently alter the single channel Po and temporal characteristics of Ca^{2+} signals evoked by agonist stimulation.

Consistent with the idea that overall regulation of IP_3R activity is altered following loss of peptide continuity, we previously reported that fragmented R1 at either the third or fourth solvent exposed regions in the coupling domain, corresponding to the activity of caspase or calpain, significantly increased the ability of R1 to induce Ca^{2+} oscillations in cells (12,41,46). Notably, we have also reported that caspase cleaved R1 exhibited significantly augmented single channel open probability (12). Here we extend this finding by showing that region-specific receptor fragmentation is a general regulatory event for all isoforms of IP_3R, including R2 and R3. Interestingly, in marked contrast to R1, proteolytic fragmentation significantly decreased single channel open probability of R2 and the ability of R2 and R3 to induce oscillatory Ca^{2+} signals in cells. How does proteolysis result in subtype-specific effects on single channel activity and the temporal pattern of stimulated Ca^{2+} signals? While

extrapolating the effects on single channel activity to the global pattern of cellular Ca^{2+} signals is challenging, a current mathematical model for the generation of Ca^{2+} oscillations suggests that the oscillation frequency is dictated by the rate at which Ca^{2+} activates and subsequently inactivates the particular IP_3R (50). We speculate that this form of regulation differs between IP_3R subtypes in the native state and is altered by proteolysis. An increase in R1 Po following cleavage might reflect an increase in susceptibility to be activated (or equally possible, a decreased sensitivity to be inactivated) by the fixed $[Ca^{2+}]$ in the patch pipette with the opposite occurring for R2. In this scenario, differentially altering the relationship between IP_3R activation and deactivation could result in changes to the oscillation period.

What is the role of IP_3R fragmentation in acute pancreatitis? We used both *in vitro* and *in vivo* rodent models with different toxic stimuli to characterize the modification of IP_3R. Consistently, IP_3R were fragmented in all tested models indicating that IP_3R fragmentation may be a general event occurring in acute pancreatitis. Our studies show that IP_3R fragmentation likely occurs at an early stage in models of acute pancreatitis. Repetitive injections of supramaximal concentrations of cerulean are widely used to induce acute pancreatitis in mice and commonly seven to twelve injections are utilized (24,35,51,52). Our data demonstrates that fragmentation of IP_3R is already initiated after the third injection. Based on these data, we speculate that IP_3R fragmentation occurs at an early stage of acute pancreatitis and may represent a protective strategy employed by the cell to limit Ca^{2+} signaling. The signaling cascade linking pathological Ca^{2+} signals to acute pancreatitis has been well characterized. Specifically, a globally sustained increase of $[Ca^{2+}]_i$ in PAC is thought to contribute to secretory inhibition, premature intracellular digestive enzyme activation, cell death, and eventually acute pancreatitis (53-55). Based on these ideas, experimental strategies aimed at inhibiting pathological Ca^{2+} signals, such as attenuating Ca^{2+} channel activity (56-58), chelating intracellular Ca^{2+} (59-61), or promoting Ca^{2+} clearance (62) have been shown to be protective in acute pancreatitis. Notably, we demonstrated that fragmented R2 and R3 may play a similar functional role, by reducing the frequency of Ca^{2+} oscillations and essentially decreasing the overall $[Ca^{2+}]_i$ in PAC in models of acute pancreatitis. Specifically, R2 and R3 fragmentation transformed sustained Ca^{2+} responses or robust elevated Ca^{2+} oscillations into lower frequency Ca^{2+} transients, and thereby would be predicted to decrease the total amount of Ca^{2+} release into the cytosol by both internal Ca^{2+} release and subsequently, store dependent Ca^{2+} influx. This process may either delay or suppress the premature intracellular digestive enzyme activation, and thereby be protective at the early stage of acute pancreatitis. In conclusion, combined with our previous report (12,45), our data is the first to systemically investigate and characterize the functional consequences of proteolytic fragmentation of all three isoforms of IP_3R. Further, to our knowledge this is the first report to show the modification of IP_3R in acute pancreatitis and provide a possible explanation for the alteration of the spatial and temporal properties of Ca^{2+} signals observed in the early stages of acute experimental pancreatitis.

Materials and Methods

Reagents

All restriction enzymes and T4-DNA ligase were from New England Biolabs. RPMI-1640 medium, penicillin/streptomycin, G418 sulfate, β-mercaptoethanol and chicken serum were purchased from Invitrogen. Fetal bovine serum was from Gemini Bio-products. Fura-2AM was from TEFLABS. Enhanced chemiluminescent substrate and ^{TM}800CW secondary anti-bodies were from Thermo Scientific. The Dc protein assay kit, Tris base, glycine, horseradish peroxidase-conjugated secondary antibodies, and all reagents used for SDS-PAGE were from Bio-Rad. Mouse anti-chicken IgM was from Southernbiotech. The antibody against the N-terminus of R2 (NT2) was generated by Pocono Rabbit Farms and Laboratories. Mouse monoclonal antibody against N-terminus of R3 (NT3) was from BD Transduction Laboratories. Both NT2 and NT3 were diluted (1:1000) for western blot. CT2 and CT3 were gifts from Richard Wojcikiewicz lab. CT2 and CT3 were raised against the extreme carboxyl aa 2686–2701 of R2 and aa 2658-2670 of R3 respectively (28). Both CT2 and CT3 were diluted (1:200) for western blot. All IP_3R antibodies used in this study have been shown to be subtype

specific based on appropriate recognition of a single isoform by western blotting cell lysates containing only a specific isoform (10,18,28,42). Cerulean and taurolithocholic acid 3-sulfate disodium salt were from Sigma-Aldrich. Gabexate Mesylate and Camostat Mesilate were from Selleckchem.

Animal Husbandry

Experiments with animal were conducted in accordance with protocol 100783 / UCAR-2001-214R approved by the University of Rochester. Mice used for this work were twelve-week-old C57/BL6NJ mice. Rats used for this work were adult male Wistar rats.

Cell Culture and Plasmid Transfection

DT40–3KO cells were grown in RPMI 1640 medium supplemented with 1% chicken serum, 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin at 39°C with 5% CO_2. DT40–3KO cell transfection and generation of stable cell lines was performed as described previously using the Amaxa nucleofector (Lonza Laboratories). DT40-3KO were generated and authenticated in the Riken institute, Japan and originally obtained directly from this source (63).

Construct Preparation

The method for generation of fragmented IP_3R was first described elsewhere in details (41). cDNA encoding R2 I-II+III-V (trypsin), R2 I-III+IV-V (trypsin), R2 I-IV+V (calpain), R3 I-II+III-V (trypsin), R3 I-III+IV-V (trypsin), and R3 I-IV+V (trypsin) were constructed using corresponding primers (forward, 5'-GATGGAAGCAACAATGTCATGAGGTAGAA TTCGCGGCCGCGCTAGCATGACCATCCACG GAGTGGGAGAGATGA-3'), (forward, 5'-ATGGGCTGGAGACTCTCAGCTCGCTAGAA TTCGCGGCGCGCTAGCATGTCTGGACCTCG CTTCAAGGAAG-3'), (forward, 5'-GGAATGAAAGGGCAGTTAACAGAATAGAA TTCGCGGCCGCGCTAGCATGGCGTCTTCAG CCACATCC-3'), (Forward 5'-CCTGGTGGCAAGAATGTGCGGAGGTAGAA TTCGCGGCCGCGCTAGCATGTCCATCCAGG GGGTGGGGCACA-3'), (Forward 5'-GCCAACTACAAGACGGCCACCAGGTAGAA TTCGCGGCCGCGCTAGCATGACCTTCCCTC GGGTCATCCCC-3'), and (5'-CACCGGGGGCACGACGTGAGCGAGTAGAA TTCGCGGCCGCGCTAGCATGCGTGCGCAG AACAACGAGATGGGG-3'), respectively. To introduce a TEV protease cleavage site after the Arg-1884 in R2, primer (5'-AGCATACTGTGTGTACAGAGAAAACCTGT ACTTCCAATCTAGAGAGATGGACCCGGAA AT-3') was used.

Native Gel Analysis

Cells were harvested by centrifugation and lysed in CHAPS lysis buffer (40 mM NaCl, 25 mM HEPES, 10 mM CHAPS, 1 mM EDTA, pH 7.4) supplemented with protease inhibitors. After 20 min on ice at 4 °C, lysates were cleared by centrifugation at 16,000 g for 10 min at 4 °C. Cleared lysates were mixed with of 4X sample buffer, 5% G-250 sample additive, and fractionated 3–12% native-PAGETM Novex gels. Separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes and probed using the indicated primary antibodies and the appropriate horseradish peroxidase-conjugated secondary antibodies. Protein bands were detected using enhanced chemiluminescent substrate.

Subcellular Fractionation

Cells were harvested and washed with ice-cold PBS, and then resuspended in homogenization buffer containing 20 mM HEPES, 5 mM NaN_3, 0.5 mM EGTA, and 320 mM sucrose (pH7.4) supplemented with protease inhibitors. Cells were homogenized using a Teflon glass homogenizer. Homogenates were cleared by centrifugation at 1000g for 10 min at 4 °C. The resulting supernatants were centrifuged at 100,000g at 4°C for 1 h. The supernatants designated as the cytosolic fraction were removed, and the microsomal pellet was resuspended in lysis buffer. Equivalent amounts of proteins were fractionated and processed for immunoblot analyses with the indicated antibodies.

Fluorescence Imaging

DT40 cells expressing defined IP_3R constructs were loaded with 2 µM Fura-2 (AM) on a glass cover-slip mounted onto a Warner chamber at room temperature for 20-30 min. Loaded cells were perfused with HEPES imaging buffer (137 mM NaCl, 4.7 mM KCl, 1.26 mM$CaCl_2$,1mM Na_2HPO_4, 0.56 mM $MgCl_2$, 10mM HEPES, 5.5 mM glucose,

pH 7.4) and stimulated with the indicated agonist. Ca^{2+} imaging was performed using an inverted epifluorescence Nikon microscope with a X40 oil immersion objective (NA=1.3). Cells were alternately excited at 340 and 380 nm, and emission was monitored at 505 nm. Images were captured every second with an exposure of 10 ms and 4 by 4 binning using a digital camera (Cooke Sensicam QE) driven by TILL Photonics software.

***In vivo* Acute Pancreatitis Model**

Twelve weeks old C57/BL6NJ mice were starved overnight and then were given three consecutive i.p. injections of cerulean (50 µg/kg/injection) hourly. Mice in the control group received the same amount of 0.9% saline instead. Mice were euthanized one hour after the third injection and pancreata were removed for further analysis. All animal procedures were approved by The University of Rochester, University Committee on Animal Resources.

Pancreatic lobule imaging using multiphoton microscopy

Mice were euthanized and pancreas were removed and placed into oxygen bubbled ice-cold imaging buffer (137 mM NaCl, 4.7 mM KCl, 1.26 mM$CaCl_2$,1mM Na_2HPO_4, 0.56 mM $MgCl_2$, 10mM HEPES, 5.5 mM glucose, pH 7.4). Pancreatic lobules were excised and exposed to liberase for 10 min followed by loading with Fluo-2 (AM). Fluo2 loaded lobules were excited at 810nm using a Spectra Physics tunable fS pulsed Ti-Sapphire laser controlled by Fluoview software on an Olympus FV1000MP microscope using a 25×water immersion objective (1.03 NA). The clusters were stimulated with various concentrations of CCh (CCh). Images were acquired at a resolution of 512 ×512 pixels. Regions of interest were selected and fluorescence intensity in that region was determined as a function of time and expressed relative to the initial fluorescence.

Isolation of Rat Pancreatic Acinar Cells

Rat pancreata were obtained from male adult Wistar rats. Pancreata were enzymatically digested with type II collagenase (Sigma) in Dulbecco's modified Eagle's medium (Invitrogen) with 0.1% bovine serum albumin and 1 mg/ml soybean trypsin inhibitor for 30 min, followed by gentle trituration. Acini were then filtered through 350 µm nylon mesh, centrifuged at 75g through 1% bovine serum albumin in Dulbecco's modified Eagle's medium, and resuspended in 1% bovine serum albumin in Dulbecco's modified Eagle's medium.

Single Channel Patch Clamp with On-nucleus Configuration

Isolated DT40 nuclei were prepared by homogenization as previously described (64). Single IP_3R channel potassium currents (ik) were measured in the on-nucleus patch clamp configuration using pCLAMP 9 and an Axopatch 200B amplifier (Molecular Devices) as previously described (64). TEV protease was included in the pipette solution for the corresponding experiments. Gigaohm seals were attained and channel activity was verified at -100 mV. Patches were then depolarized to 0 mV for 15 minutes to allow TEV protease to cleave the receptor. The patches were again repolarized to -100 mV and channel activity was measured. Traces were consecutive 3 second sweeps, sampled at 20 kHz and filtered at 5 kHz. Pipette resistances were typically 20 MΩ and seal resistances were >5 GΩ.

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Conflict of interest:
The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions:

This work was performed in the Department of Pharmacology and Physiology at the University of Rochester. L. W. designed and performed experiments, collected and analyzed the data (Figure 1-8), drafted the manuscript, and prepared all the figures. L. E. W. collected and analyzed data obtained through single-channel electrophysiology and prepared the figure (Figure 9). K. J. A. designed and expressed some of the constructs and drafted the manuscript. D. I. Y. was responsible for the conception and design of all experiments as well as data analysis, generation of figures, and editing of the manuscript. All authors approved the final version.

References

1. Foskett, J. K., White, C., Cheung, K. H., and Mak, D. O. (2007) Inositol trisphosphate receptor Ca2+ release channels. *Physiol Rev* **87**, 593-658
2. Yule, D. I., Betzenhauser, M. J., and Joseph, S. K. (2010) Linking structure to function: Recent lessons from inositol 1,4,5-trisphosphate receptor mutagenesis. *Cell calcium* **47**, 469-479
3. Alzayady, K. J., Sebe-Pedros, A., Chandrasekhar, R., Wang, L., Ruiz-Trillo, I., and Yule, D. I. (2015) Tracing the Evolutionary History of Inositol, 1, 4, 5-Trisphosphate Receptor: Insights from Analyses of Capsaspora owczarzaki Ca2+ Release Channel Orthologs. *Mol Biol Evol* **32**, 2236-2253
4. Wang, R. C. D. I. Y. a. L. (2017) Inositol 1,4,5-trisphosphate receptors (InsP3R). in *PANCREAPEDIA (Exocrine Pancreas Knowledge Base)*, October 19 Ed.
5. Berridge, M. J. (2009) Inositol trisphosphate and calcium signalling mechanisms. *Biochimica et biophysica acta* **1793**, 933-940
6. Berridge, M. J. (2016) The Inositol Trisphosphate/Calcium Signaling Pathway in Health and Disease. *Physiol Rev* **96**, 1261-1296
7. Luyten, T., Welkenhuyzen, K., Roest, G., Kania, E., Wang, L., Bittremieux, M., Yule, D. I., Parys, J. B., and Bultynck, G. (2017) Resveratrol-induced autophagy is dependent on IP3Rs and on cytosolic Ca2. *Biochimica et biophysica acta*
8. Chandrasekhar, R., Alzayady, K. J., Wagner, L. E., 2nd, and Yule, D. I. (2016) Unique Regulatory Properties of Heterotetrameric Inositol 1,4,5-Trisphosphate Receptors Revealed by Studying Concatenated Receptor Constructs. *The Journal of biological chemistry* **291**, 4846-4860
9. Patterson, R. L., Boehning, D., and Snyder, S. H. (2004) Inositol 1,4,5-trisphosphate receptors as signal integrators. *Annu Rev Biochem* **73**, 437-465
10. Alzayady, K. J., Wang, L., Chandrasekhar, R., Wagner, L. E., 2nd, Van Petegem, F., and Yule, D. I. (2016) Defining the stoichiometry of inositol 1,4,5-trisphosphate binding required to initiate Ca2+ release. *Sci Signal* **9**, ra35
11. Wright, F. A., and Wojcikiewicz, R. J. (2016) Chapter 4 - Inositol 1,4,5-Trisphosphate Receptor Ubiquitination. *Prog Mol Biol Transl Sci* **141**, 141-159
12. Wang, L., Wagner, L. E., 2nd, Alzayady, K. J., and Yule, D. I. (2017) Region-specific proteolysis differentially regulates type 1 inositol 1,4,5-trisphosphate receptor activity. *The Journal of biological chemistry* **292**, 11714-11726
13. Futatsugi, A., Nakamura, T., Yamada, M. K., Ebisui, E., Nakamura, K., Uchida, K., Kitaguchi, T., Takahashi-Iwanaga, H., Noda, T., Aruga, J., and Mikoshiba, K. (2005) IP3 receptor types 2 and 3 mediate exocrine secretion underlying energy metabolism. *Science* **309**, 2232-2234
14. Fujino, I., Yamada, N., Miyawaki, A., Hasegawa, M., Furuichi, T., and Mikoshiba, K. (1995) Differential expression of type 2 and type 3 inositol 1,4,5-trisphosphate receptor mRNAs in various mouse tissues: in situ hybridization study. *Cell Tissue Res* **280**, 201-210
15. Teos, L. Y., Zhang, Y., Cotrim, A. P., Swaim, W., Won, J. H., Ambrus, J., Shen, L., Bebris, L., Grisius, M., Jang, S. I., Yule, D. I., Ambudkar, I. S., and Alevizos, I. (2015) IP3R deficit underlies loss of salivary fluid secretion in Sjogren's Syndrome. *Sci Rep* **5**, 13953
16. Bezprozvanny, I. (2005) The inositol 1,4,5-trisphosphate receptors. *Cell calcium* **38**, 261-272
17. Diaz, F., and Bourguignon, L. Y. (2000) Selective down-regulation of IP(3)receptor subtypes by caspases and calpain during TNF alpha -induced apoptosis of human T-lymphoma cells. *Cell calcium* **27**, 315-328
18. Wojcikiewicz, R. J., Ernst, S. A., and Yule, D. I. (1999) Secretagogues cause ubiquitination and down-regulation of inositol 1, 4,5-trisphosphate receptors in rat pancreatic acinar cells. *Gastroenterology* **116**, 1194-1201

19. Maes, K., Missiaen, L., Parys, J. B., De Smet, P., Sienaert, I., Waelkens, E., Callewaert, G., and De Smedt, H. (2001) Mapping of the ATP-binding sites on inositol 1,4,5-trisphosphate receptor type 1 and type 3 homotetramers by controlled proteolysis and photoaffinity labeling. *The Journal of biological chemistry* **276**, 3492-3497
20. Soulsby, M. D., and Wojcikiewicz, R. J. (2005) The type III inositol 1,4,5-trisphosphate receptor is phosphorylated by cAMP-dependent protein kinase at three sites. *The Biochemical journal* **392**, 493-497
21. Yoshikawa, F., Iwasaki, H., Michikawa, T., Furuichi, T., and Mikoshiba, K. (1999) Trypsinized cerebellar inositol 1,4,5-trisphosphate receptor. Structural and functional coupling of cleaved ligand binding and channel domains. *The Journal of biological chemistry* **274**, 316-327
22. Adler, G., Rohr, G., and Kern, H. F. (1982) Alteration of membrane fusion as a cause of acute pancreatitis in the rat. *Dig Dis Sci* **27**, 993-1002
23. Lerch, M. M., and Gorelick, F. S. (2013) Models of acute and chronic pancreatitis. *Gastroenterology* **144**, 1180-1193
24. Ward, J. B., Sutton, R., Jenkins, S. A., and Petersen, O. H. (1996) Progressive disruption of acinar cell calcium signaling is an early feature of cerulein-induced pancreatitis in mice. *Gastroenterology* **111**, 481-491
25. Mooren, F., Hlouschek, V., Finkes, T., Turi, S., Weber, I. A., Singh, J., Domschke, W., Schnekenburger, J., Kruger, B., and Lerch, M. M. (2003) Early changes in pancreatic acinar cell calcium signaling after pancreatic duct obstruction. *The Journal of biological chemistry* **278**, 9361-9369
26. Voronina, S., Collier, D., Chvanov, M., Middlehurst, B., Beckett, A. J., Prior, I. A., Criddle, D. N., Begg, M., Mikoshiba, K., Sutton, R., and Tepikin, A. V. (2015) The role of Ca2+ influx in endocytic vacuole formation in pancreatic acinar cells. *The Biochemical journal* **465**, 405-412
27. De Smedt, H., Missiaen, L., Parys, J. B., Henning, R. H., Sienaert, I., Vanlingen, S., Gijsens, A., Himpens, B., and Casteels, R. (1997) Isoform diversity of the inositol trisphosphate receptor in cell types of mouse origin. *The Biochemical journal* **322 (Pt 2)**, 575-583
28. Wojcikiewicz, R. J. (1995) Type I, II, and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. *The Journal of biological chemistry* **270**, 11678-11683
29. Sherwood, M. W., Prior, I. A., Voronina, S. G., Barrow, S. L., Woodsmith, J. D., Gerasimenko, O. V., Petersen, O. H., and Tepikin, A. V. (2007) Activation of trypsinogen in large endocytic vacuoles of pancreatic acinar cells. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 5674-5679
30. Saluja, A. K., Donovan, E. A., Yamanaka, K., Yamaguchi, Y., Hofbauer, B., and Steer, M. L. (1997) Cerulein-induced in vitro activation of trypsinogen in rat pancreatic acini is mediated by cathepsin B. *Gastroenterology* **113**, 304-310
31. Hofbauer, B., Saluja, A. K., Lerch, M. M., Bhagat, L., Bhatia, M., Lee, H. S., Frossard, J. L., Adler, G., and Steer, M. L. (1998) Intra-acinar cell activation of trypsinogen during caerulein-induced pancreatitis in rats. *Am J Physiol* **275**, G352-362
32. Balldin, G., and Ohlsson, K. (1979) Demonstration of pancreatic protease-antiprotease complexes in the peritoneal fluid of patients with acute pancreatitis. *Surgery* **85**, 451-456
33. Joseph, S. K., Pierson, S., and Samanta, S. (1995) Trypsin digestion of the inositol trisphosphate receptor: implications for the conformation and domain organization of the protein. *Biochem J* **307 (Pt 3)**, 859-865
34. Betzenhauser, M. J., Fike, J. L., Wagner, L. E., 2nd, and Yule, D. I. (2009) Protein kinase A increases type-2 inositol 1,4,5-trisphosphate receptor activity by phosphorylation of serine 937. *The Journal of biological chemistry* **284**, 25116-25125

35. Dawra, R., Sah, R. P., Dudeja, V., Rishi, L., Talukdar, R., Garg, P., and Saluja, A. K. (2011) Intra-acinar trypsinogen activation mediates early stages of pancreatic injury but not inflammation in mice with acute pancreatitis. *Gastroenterology* **141**, 2210-2217 e2212
36. Williams, J. A., Korc, M., and Dormer, R. L. (1978) Action of secretagogues on a new preparation of functionally intact, isolated pancreatic acini. *Am J Physiol* **235**, 517-524
37. Chaudhuri, A., Kolodecik, T. R., and Gorelick, F. S. (2005) Effects of increased intracellular cAMP on carbachol-stimulated zymogen activation, secretion, and injury in the pancreatic acinar cell. *Am J Physiol Gastrointest Liver Physiol* **288**, G235-243
38. Voronina, S., Longbottom, R., Sutton, R., Petersen, O. H., and Tepikin, A. (2002) Bile acids induce calcium signals in mouse pancreatic acinar cells: implications for bile-induced pancreatic pathology. *J Physiol* **540**, 49-55
39. Muili, K. A., Wang, D., Orabi, A. I., Sarwar, S., Luo, Y., Javed, T. A., Eisses, J. F., Mahmood, S. M., Jin, S., Singh, V. P., Ananthanaravanan, M., Perides, G., Williams, J. A., Molkentin, J. D., and Husain, S. Z. (2013) Bile acids induce pancreatic acinar cell injury and pancreatitis by activating calcineurin. *The Journal of biological chemistry* **288**, 570-580
40. Muili, K. A., Jin, S., Orabi, A. I., Eisses, J. F., Javed, T. A., Le, T., Bottino, R., Jayaraman, T., and Husain, S. Z. (2013) Pancreatic acinar cell nuclear factor kappaB activation because of bile acid exposure is dependent on calcineurin. *The Journal of biological chemistry* **288**, 21065-21073
41. Alzayady, K. J., Chandrasekhar, R., and Yule, D. I. (2013) Fragmented inositol 1,4,5-trisphosphate receptors retain tetrameric architecture and form functional Ca2+ release channels. *The Journal of biological chemistry* **288**, 11122-11134
42. Betzenhauser, M. J., Wagner, L. E., 2nd, Iwai, M., Michikawa, T., Mikoshiba, K., and Yule, D. I. (2008) ATP modulation of Ca2+ release by type-2 and type-3 inositol (1, 4, 5)-triphosphate receptors. Differing ATP sensitivities and molecular determinants of action. *The Journal of biological chemistry* **283**, 21579-21587
43. Alzayady, K. J., Wagner, L. E., 2nd, Chandrasekhar, R., Monteagudo, A., Godiska, R., Tall, G. G., Joseph, S. K., and Yule, D. I. (2013) Functional inositol 1,4,5-trisphosphate receptors assembled from concatenated homo- and heteromeric subunits. *The Journal of biological chemistry* **288**, 29772-29784
44. Miyakawa, T., Maeda, A., Yamazawa, T., Hirose, K., Kurosaki, T., and Iino, M. (1999) Encoding of Ca2+ signals by differential expression of IP3 receptor subtypes. *The EMBO journal* **18**, 1303-1308
45. Wang, L., Alzayady, K. J., and Yule, D. I. (2016) Proteolytic fragmentation of inositol 1,4,5-trisphosphate receptors: a novel mechanism regulating channel activity? *J Physiol* **594**, 2867-2876
46. Sneyd, J., Han, J. M., Wang, L., Chen, J., Yang, X., Tanimura, A., Sanderson, M. J., Kirk, V., and Yule, D. I. (2017) On the dynamical structure of calcium oscillations. *Proceedings of the National Academy of Sciences of the United States of America* **114**, 1456-1461
47. Hirota, J., Furuichi, T., and Mikoshiba, K. (1999) Inositol 1,4,5-trisphosphate receptor type 1 is a substrate for caspase-3 and is cleaved during apoptosis in a caspase-3-dependent manner. *J Biol Chem* **274**, 34433-34437
48. Kopil, C. M., Vais, H., Cheung, K. H., Siebert, A. P., Mak, D. O., Foskett, J. K., and Neumar, R. W. (2011) Calpain-cleaved type 1 inositol 1,4,5-trisphosphate receptor (InsP(3)R1) has InsP(3)-independent gating and disrupts intracellular Ca(2+) homeostasis. *J Biol Chem* **286**, 35998-36010
49. Fan, G., Baker, M. L., Wang, Z., Baker, M. R., Sinyagovskiy, P. A., Chiu, W., Ludtke, S. J., and Serysheva, II. (2015) Gating machinery of InsP3R channels revealed by electron cryomicroscopy. *Nature* **527**, 336-341
50. Hille, B. (2001) *Ionic channels of excitable membranes*, Sinaur Associates, Sunderland, Mass.

51. Kaiser, A. M., Saluja, A. K., Sengupta, A., Saluja, M., and Steer, M. L. (1995) Relationship between severity, necrosis, and apoptosis in five models of experimental acute pancreatitis. *Am J Physiol* **269**, C1295-1304
52. Gukovskaya, A. S., Vaquero, E., Zaninovic, V., Gorelick, F. S., Lusis, A. J., Brennan, M. L., Holland, S., and Pandol, S. J. (2002) Neutrophils and NADPH oxidase mediate intrapancreatic trypsin activation in murine experimental acute pancreatitis. *Gastroenterology* **122**, 974-984
53. Sah, R. P., Garg, P., and Saluja, A. K. (2012) Pathogenic mechanisms of acute pancreatitis. *Curr Opin Gastroenterol* **28**, 507-515
54. Pandol, S. J., Saluja, A. K., Imrie, C. W., and Banks, P. A. (2007) Acute pancreatitis: bench to the bedside. *Gastroenterology* **132**, 1127-1151
55. Saluja, A. K., Lerch, M. M., Phillips, P. A., and Dudeja, V. (2007) Why does pancreatic overstimulation cause pancreatitis? *Annu Rev Physiol* **69**, 249-269
56. Wen, L., Voronina, S., Javed, M. A., Awais, M., Szatmary, P., Latawiec, D., Chvanov, M., Collier, D., Huang, W., Barrett, J., Begg, M., Stauderman, K., Roos, J., Grigoryev, S., Ramos, S., Rogers, E., Whitten, J., Velicelebi, G., Dunn, M., Tepikin, A. V., Criddle, D. N., and Sutton, R. (2015) Inhibitors of ORAI1 Prevent Cytosolic Calcium-Associated Injury of Human Pancreatic Acinar Cells and Acute Pancreatitis in 3 Mouse Models. *Gastroenterology* **149**, 481-492 e487
57. Huang, W., Cane, M. C., Mukherjee, R., Szatmary, P., Zhang, X., Elliott, V., Ouyang, Y., Chvanov, M., Latawiec, D., Wen, L., Booth, D. M., Haynes, A. C., Petersen, O. H., Tepikin, A. V., Criddle, D. N., and Sutton, R. (2017) Caffeine protects against experimental acute pancreatitis by inhibition of inositol 1,4,5-trisphosphate receptor-mediated Ca2+ release. *Gut* **66**, 301-313
58. Husain, S. Z., Prasad, P., Grant, W. M., Kolodecik, T. R., Nathanson, M. H., and Gorelick, F. S. (2005) The ryanodine receptor mediates early zymogen activation in pancreatitis. *Proceedings of the National Academy of Sciences of the United States of America* **102**, 14386-14391
59. Saluja, A. K., Bhagat, L., Lee, H. S., Bhatia, M., Frossard, J. L., and Steer, M. L. (1999) Secretagogue-induced digestive enzyme activation and cell injury in rat pancreatic acini. *Am J Physiol* **276**, G835-842
60. Kruger, B., Albrecht, E., and Lerch, M. M. (2000) The role of intracellular calcium signaling in premature protease activation and the onset of pancreatitis. *Am J Pathol* **157**, 43-50
61. Raraty, M., Ward, J., Erdemli, G., Vaillant, C., Neoptolemos, J. P., Sutton, R., and Petersen, O. H. (2000) Calcium-dependent enzyme activation and vacuole formation in the apical granular region of pancreatic acinar cells. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 13126-13131
62. Criddle, D. N., Murphy, J., Fistetto, G., Barrow, S., Tepikin, A. V., Neoptolemos, J. P., Sutton, R., and Petersen, O. H. (2006) Fatty acid ethyl esters cause pancreatic calcium toxicity via inositol trisphosphate receptors and loss of ATP synthesis. *Gastroenterology* **130**, 781-793
63. Sugawara, H., Kurosaki, M., Takata, M., and Kurosaki, T. (1997) Genetic evidence for involvement of type 1, type 2 and type 3 inositol 1,4,5-trisphosphate receptors in signal transduction through the B-cell antigen receptor. *The EMBO journal* **16**, 3078-3088
64. Wagner, L. E., 2nd, and Yule, D. I. (2012) Differential regulation of the InsP(3) receptor type-1 and -2 single channel properties by InsP(3), Ca(2)(+) and ATP. *J Physiol* **590**, 3245-3259

Figure legends

Figure 1. Altered temporal Ca^{2+} release profile in pancreatic lobules in the *in vivo* acute pancreatitis model. Pancreatic lobules were excised from mice given 3 consecutive i.p. injections of either 0.9% saline or caerulein (50 μg/kg/injection) followed by loading with Ca^{2+} indicator fluo-2 AM (25 μM) for one hour. $[Ca^{2+}]_i$ dynamics were measured using multi-photon microscope. PAC from mice treated with saline elicited

robust Ca^{2+} oscillations in response to 300 nM carbachol (A and D). In contrast to the control, saline injected condition, PAC from mice subjected to caerulein treatment, elicited Ca^{2+} transients with significant lower frequency (B and D), which could not be rescued by increasing the concentration of the stimulus (C and D). Experiments for each condition were repeated in preparations from three different animals with more than 20 calcium responses analyzed in each repeat. Two representative traces (black and red) were given for each of the conditions. *, statistical significance determined by one-way ANOVA followed by Tukey's multiple comparison test.

Figure 2. Generation of fragmented R2 and R3 in the *in vivo* acute pancreatitis model. Mice received three injections of saline or caerulein hourly. Pancreata were then removed, homogenized, and prepared for western blot detection. Samples from DT40-3KO cells stable expressing R2 I-III and R2 I-IV, or R3 I-III and R3 I-IV were run on the same gels to indicate the relative sizes of the fragmented receptors. R2 and R3 were fragmented in the pancreata from mice treated with caerulein (A and F). Statistics showed that there was a significant reduction of the full-length receptors (B and G) and a concomitant substantial increase of the fragmented receptors (B-D, G-I). The N-terminal fragments of R2 were co-immunoprecipitated with the C-terminal fragments using the C-terminal R2 antibody (CT2) suggesting that the receptor remain associated after proteolysis (E). Each experiment was repeated four times. Arrows in (A), (E) and (F) indicate major receptor fragments. *, statistical significance determined by student's t-test.

Figure 3. Generation of DT40-3KO cells stably expressing various types of fragmented R2 and R3. (A) and (D) show tryptic fragmentation sites (R-919, R-1583, R-1884) and the predicted calpain fragmentation site (E-1869) on the R2, and tryptic fragmentation sites (R-915, R-1574, R-1856, R-1869) on the R3. The epitopes for the R2 N-terminal antibody NT2 (aa 320-338), R2 C-terminal antibody CT2 (aa 2686-2701), R3 N-terminal antibody NT3 (aa 22-230), and R3 C-terminal antibody (aa 2658-2670) are labeled. cDNA encoding complementary pairs of polypeptide chains corresponding to various types of fragmented R2 and R3 were stably transfected into DT40-3KO cells. Western blot assays confirmed the expressions of both the N-terminal fragment and the complementary C-terminal fragment of each type of fragmented R2 (B and C) or fragmented R3 (E and F) in DT40-3KO cells.

Figure 4. Fragmentation of R2 and R3 in T7 Mice. T7 mice received three injections of saline or caerulein hourly. Pancreata were then removed, homogenized, and prepared for western blot detection. While the ratio of the amount of R2 fragments to R2 full length was still significantly increased (A and C), knockdown of trypsinogen-7 completely blocked the increase of the ratio for R3 (B and D). Arrows in (A) indicate the major receptor fragments. *, statistical significance determined by student's t-test.

Figure 5. Generation of fragmented R2 and R3 in the *in vitro* acute pancreatitis model. Rat pancreatic acinar cells were isolated and incubated with various supramaximal concentrations of carbachol for different amount of time. Cells were then harvested and subjected to western blot detection. Both R2 and R3 were cleaved in the *in vitro* acute pancreatitis model (A and B). The Bile salt, taurolithocholic acid 3-sulfate disodium salt (TLCS), also resulted in receptor fragmentation, which can be blocked by pre-incubation of PAC with trypsin inhibitors (2 μM Gabaxeate and 3 μM Camostate) (C and D). Total protein staining was performed as loading control for figure C and D in (E). Each experiment was repeated three times.

Figure 6. R2 and R3 retain tetrameric architecture and ER membrane localization after receptor fragmentation. Isolated rat PAC were incubated with various supramaximal concentrations of carbachol for different amount of time followed by membrane fractionation. Full-length receptors, the N-terminal receptor fragments, and the C-terminal receptor fragments all remained membrane associated for both R2 (A) and R3 (B). Further, native gel analysis showed that fragmented R2 and R3 migrated at same molecular weight as full-length receptors indicating that R2 and R3 retain tetrameric architecture after receptor

fragmentation (C and D). R3 I-V represents the migration pattern of dissociated monomeric N-terminal fragment. Each experiment was repeated three times.

Figure 7. All fragmented R2 and R3 were functional. A panel of DT40-3KO cells, each of which stably expressing one pair of complementary polypeptides representing fragmented R2 or R3 at particular sites, were loaded with fura-2 (AM) followed by stimulation of PAR2 receptor activation with trypsin. Single cell imaging assays showed that all types of fragmented R2 (A-D) and fragmented R3 (E-H) were capable of eliciting Ca^{2+} response in response to PAR2 activation. Each experiment was repeated more than three times with > 30 cells in each run.

Figure 8. Region specific fragmentation alters the temporal Ca^{2+} release profile of R2 and R3. A panel of DT40-3KO cells, each of which stably expressing a pair of complementary polypeptides representing fragmented R2 or R3 at particular sites, were loaded with fura-2 (AM) followed by stimulation of B-cell receptor activation with anti-IgM. Single cell Ca^{2+} imaging recording showed that fragmentation at the proximal N-terminal solvent exposed region had no impact on the frequency of Ca^{2+} oscillations mediated by R2 (A, B and E) and R3 (G, H and K). However, receptor fragmentation more toward to the C-terminus resulted in a significant decrease in Ca^{2+} oscillations mediated by fragmented R2 (C-E) and fragmented R3 (I-K). This decrease in the frequency of Ca^{2+} oscillations could not be rescued by increasing the concentrations of stimuli (F and L). *, statistical significance determined by one-way ANOVA followed by Tukey's multiple comparison test.

Figure 9. Receptor fragmentation alters R2 activity at the single channel level. A TEV protease cleavage sequence was inserted into R2 to achieve a specific receptor cleavage at the fourth solvent exposed region. TEV protease neither cleaved the R2 WT nor had an impact on its channel activity (A, B, E-G). In contrast, TEV protease specifically cleaved the R2 (TEV) at the predicted site and significantly decreased channel open probability and increased interburst interval with no impact on burst length (C-G). NT2 antibody was used to detect R2 in the western blot assays. Each experimental condition was repeated five times. *, statistical significance determined by one-way ANOVA followed by Tukey's multiple comparison test.

Figure 1

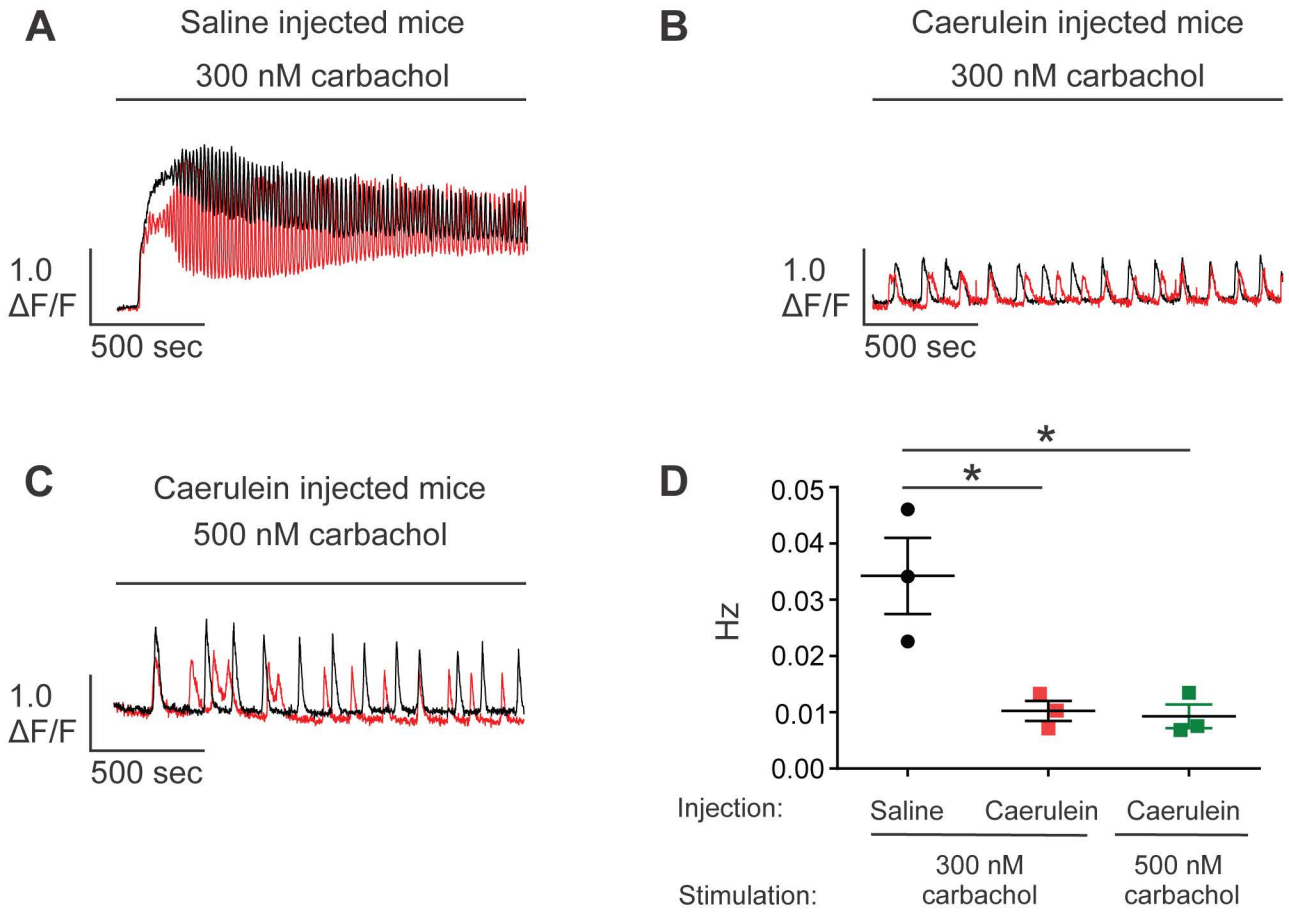
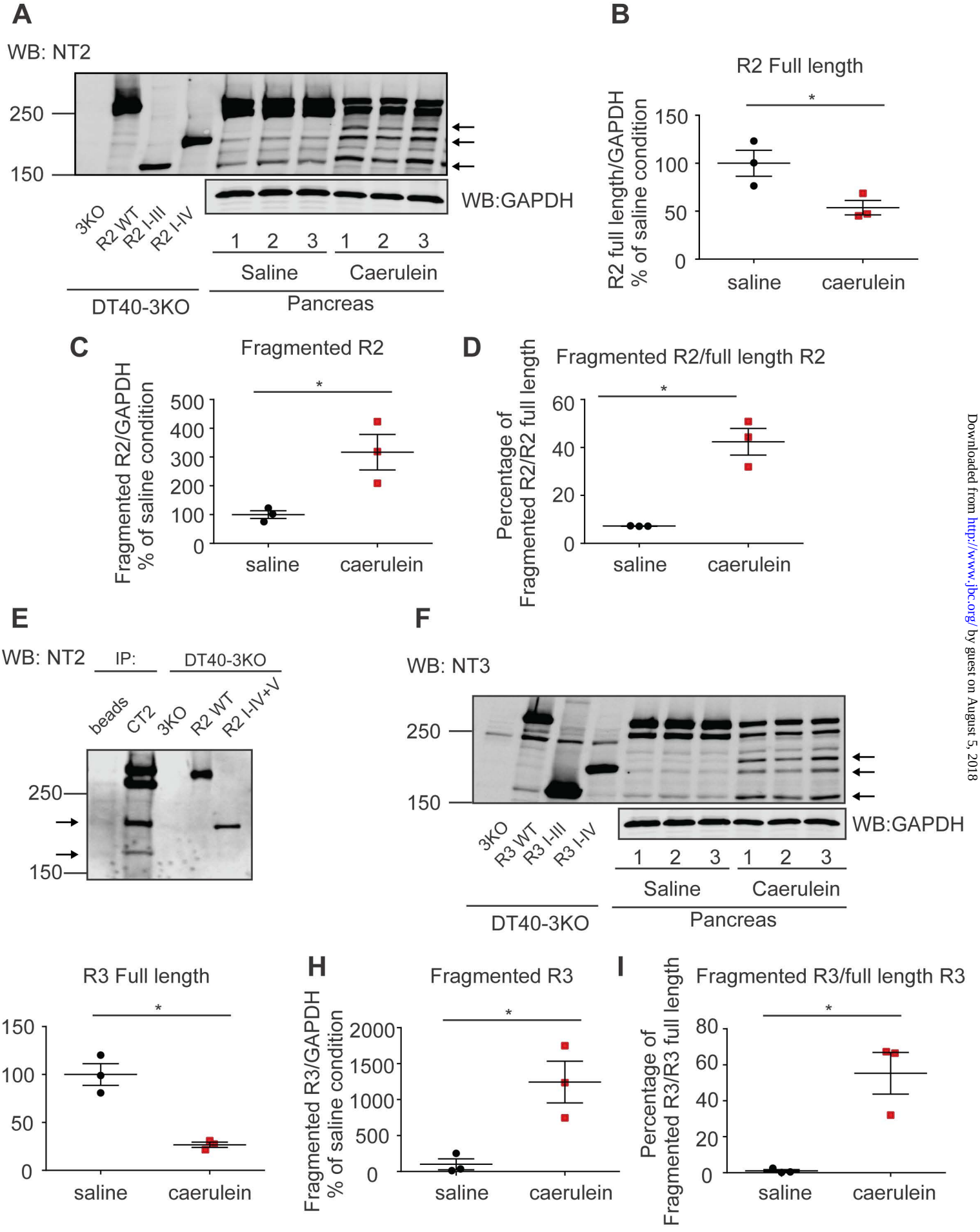


Figure 2



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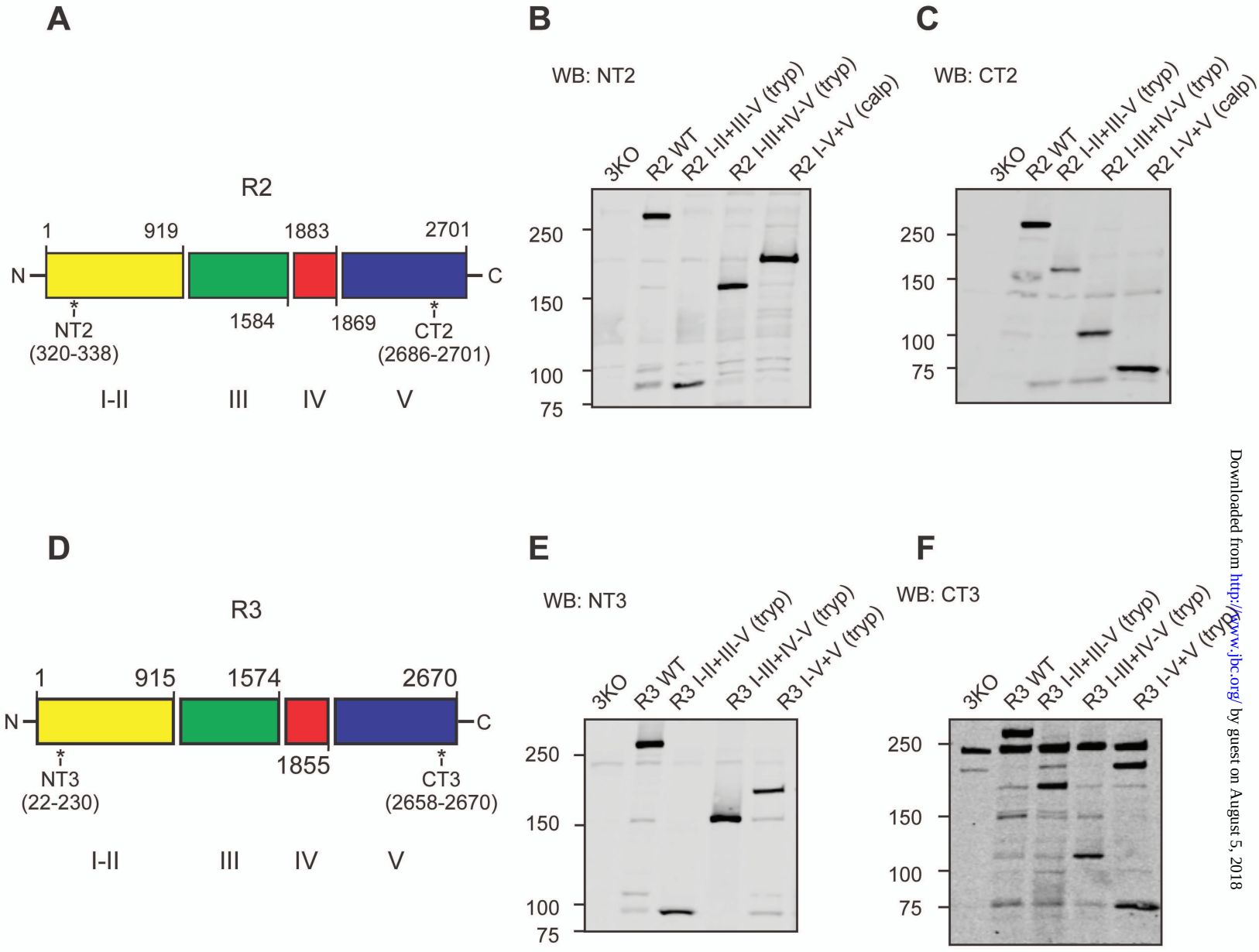


Figure 4

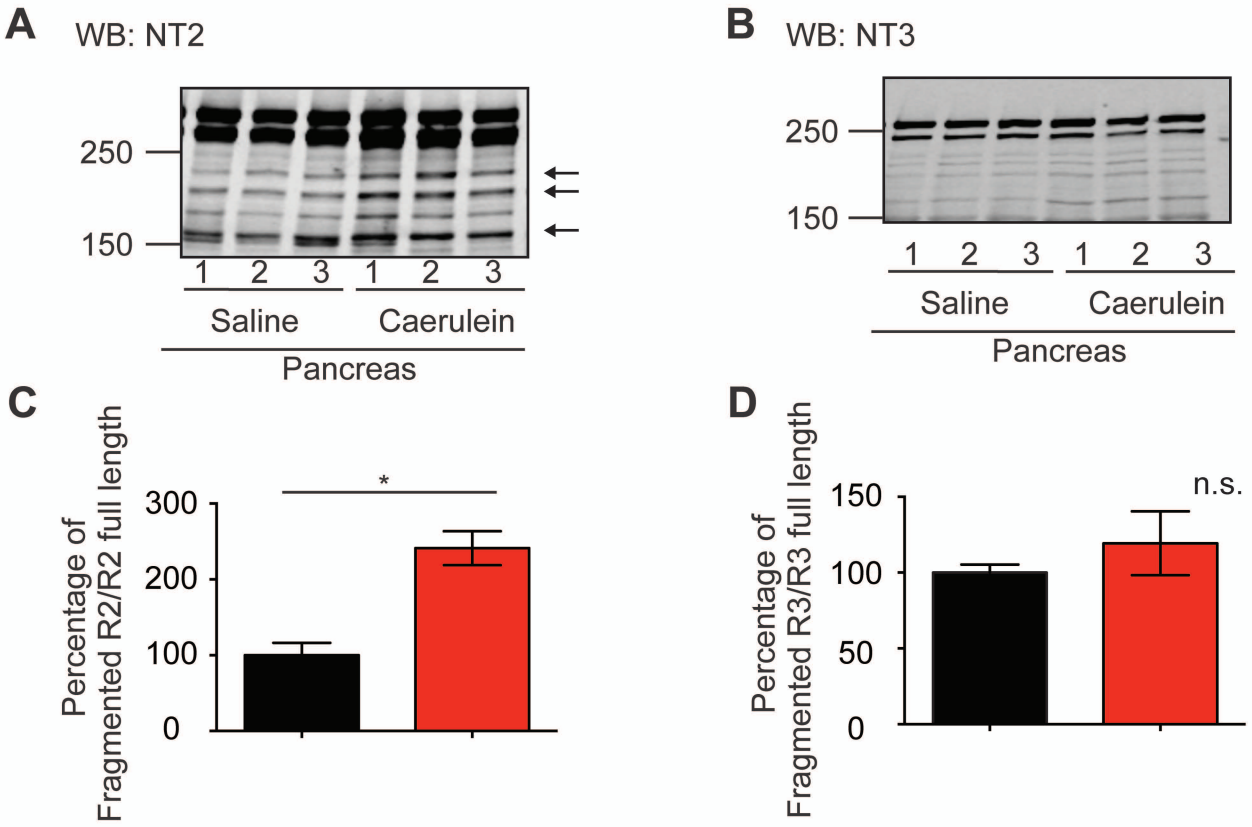


Figure 5

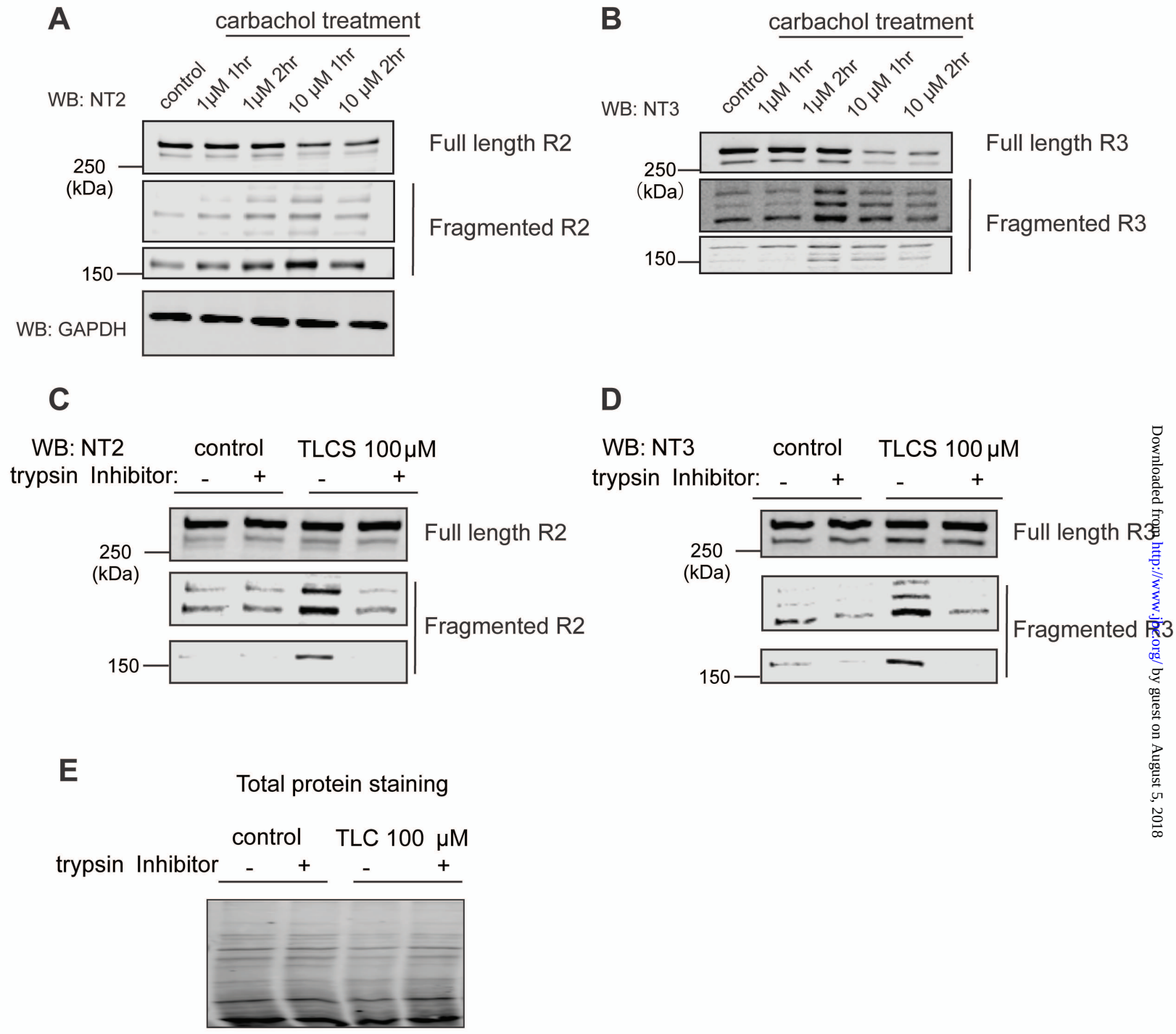


Figure 6

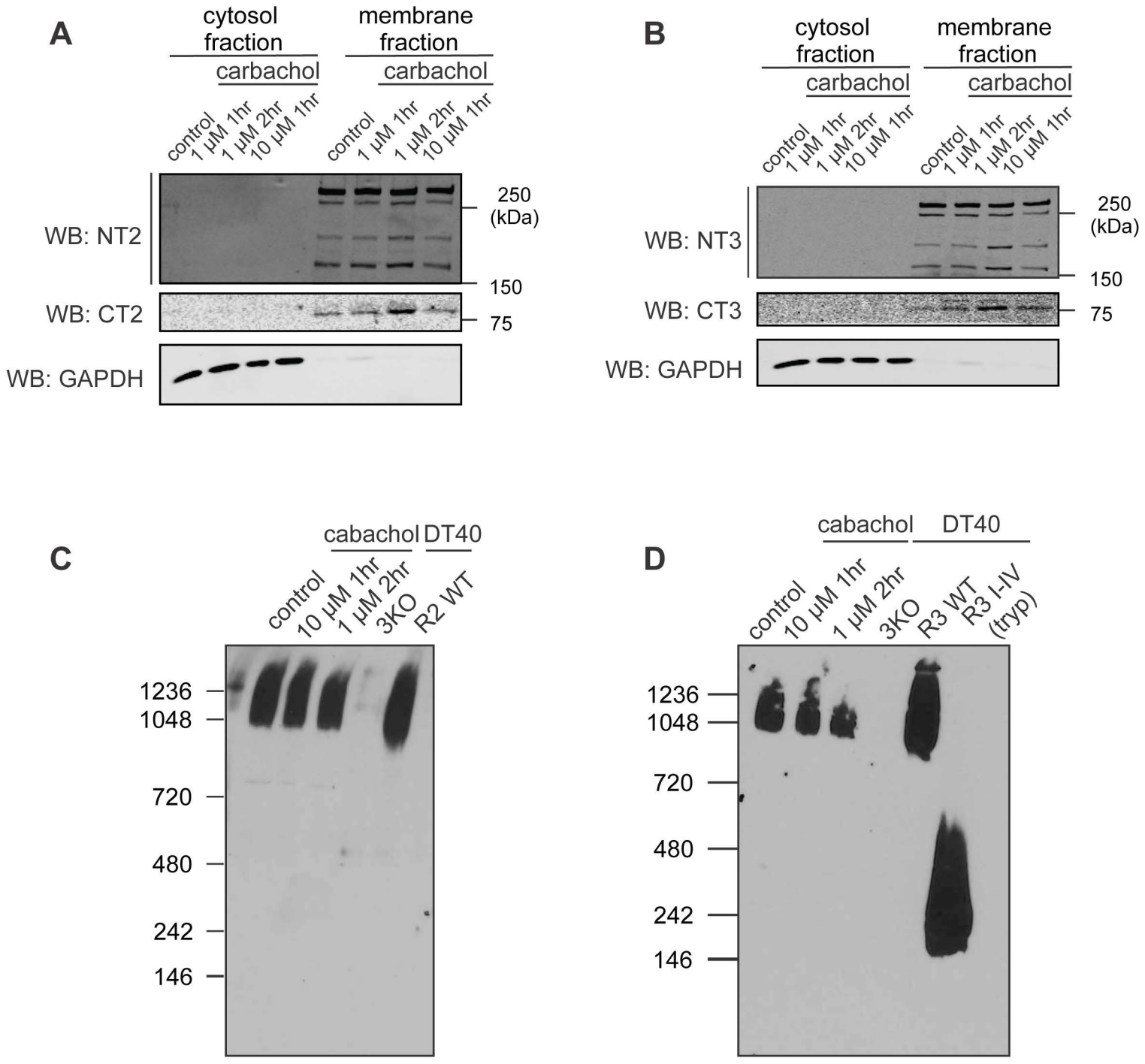


Figure 7

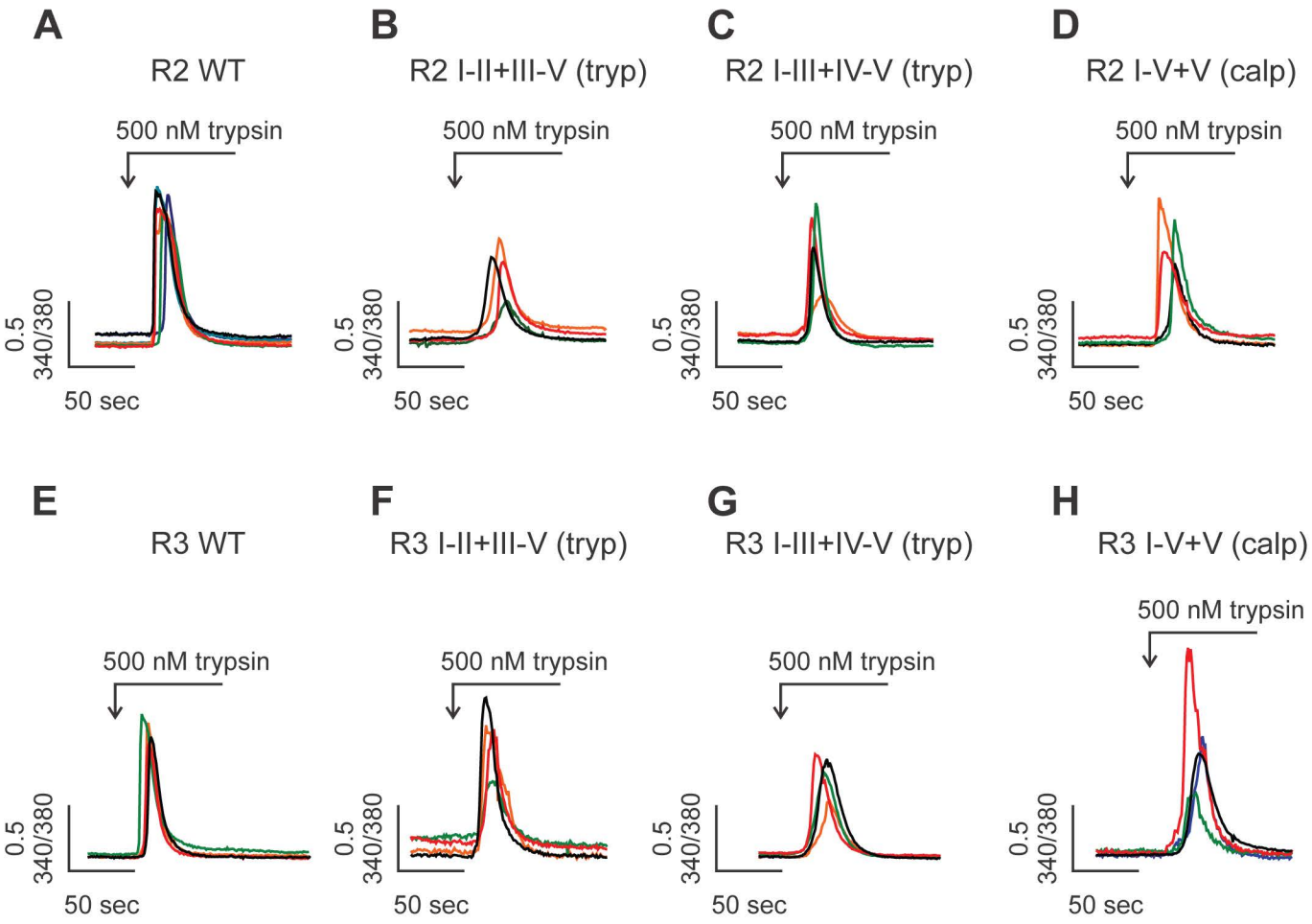


Figure 8

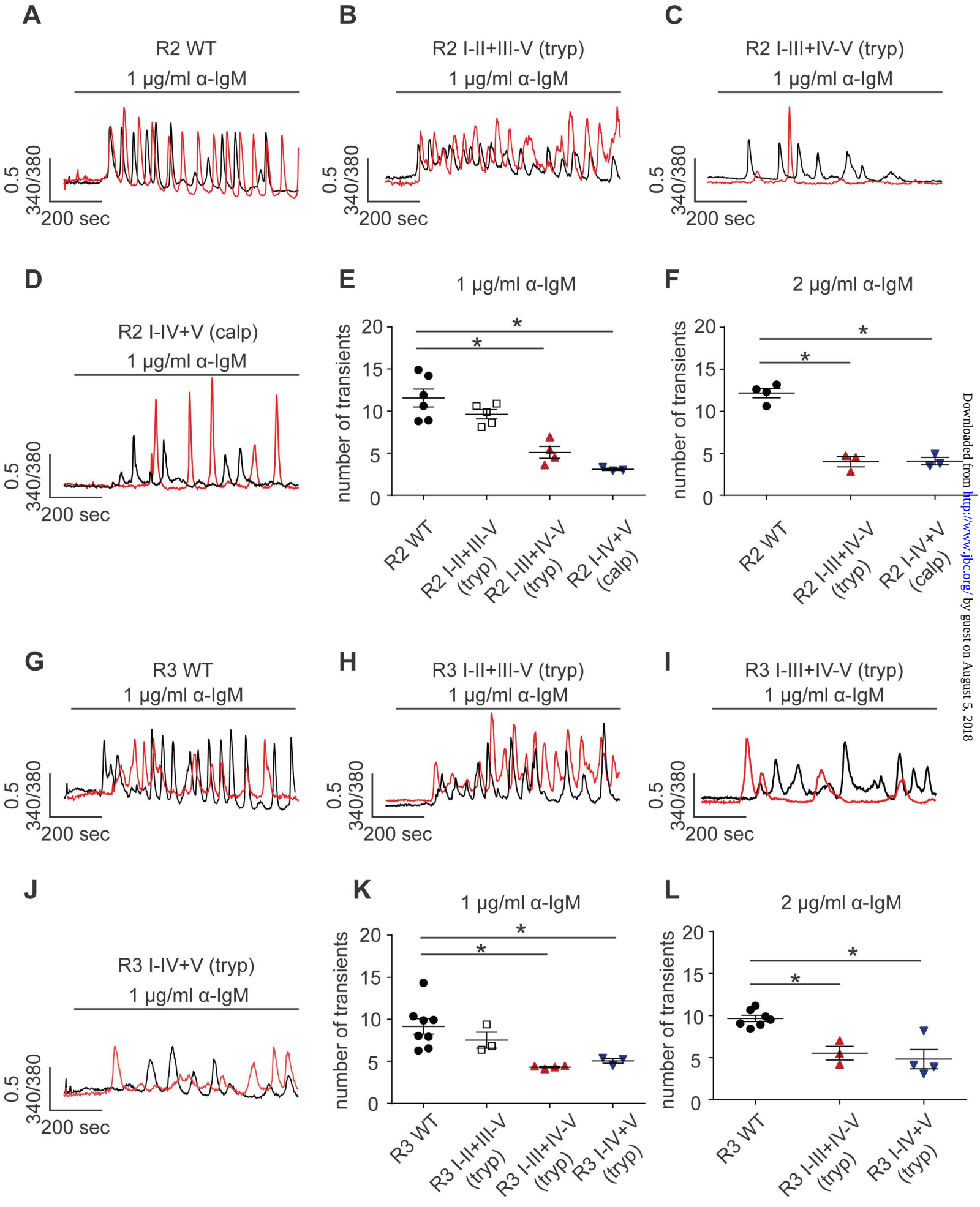
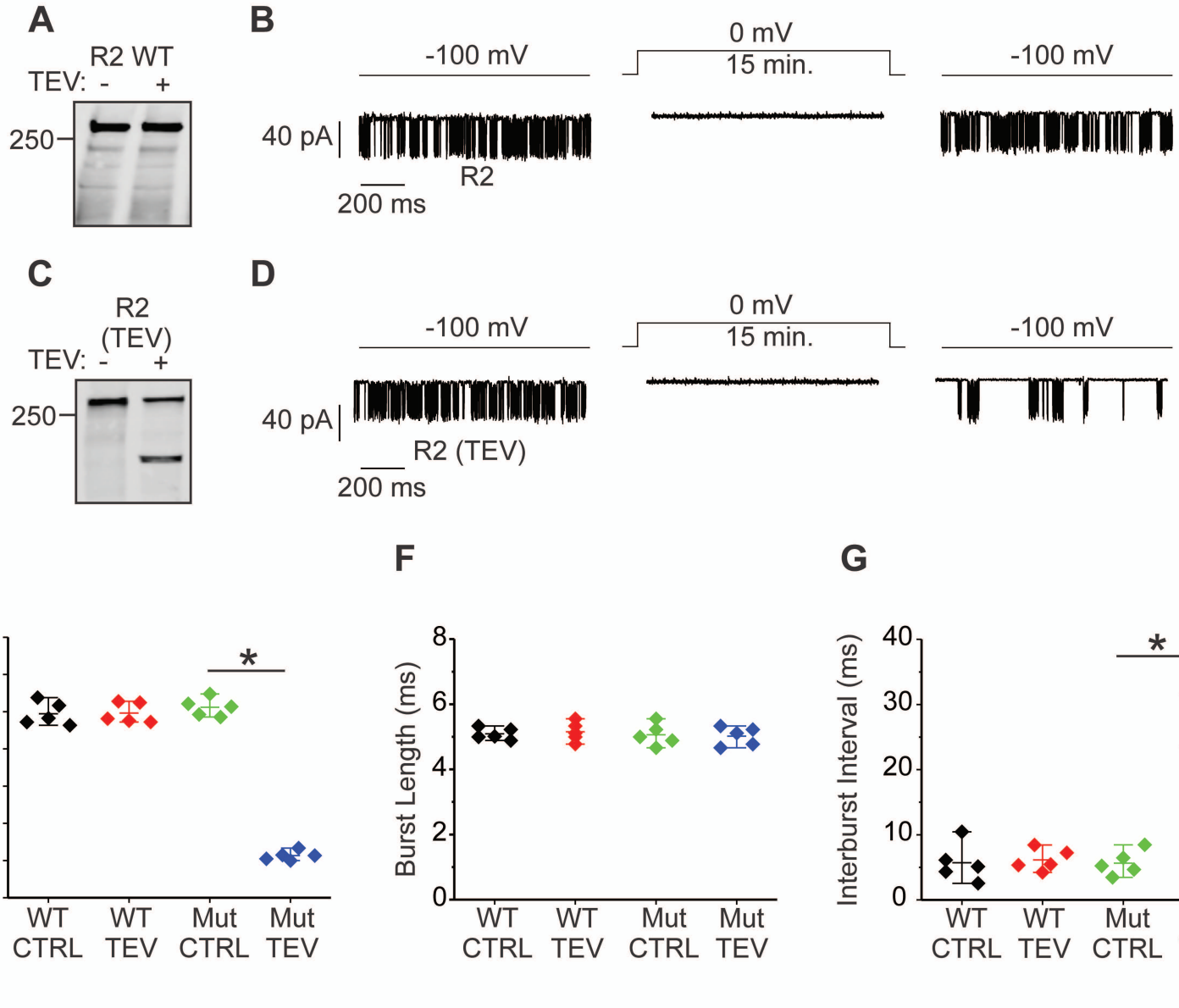


Figure 9



Region-specific proteolysis differentially modulates type 2 and type 3 inositol 1,4,5-trisphosphate receptor activity in models of acute pancreatitis

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