JPT2/HN1L functions as an NAADP-binding protein in a cell-type specific manner

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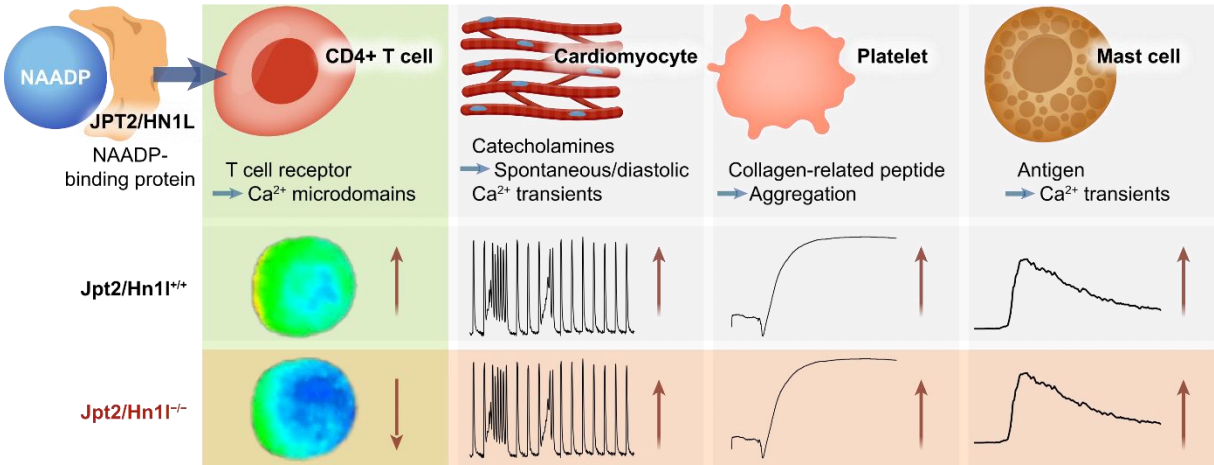
Graphical abstract



Highlights

• JPT2/HN1L has been identified as an NAADP-binding protein mediating NAADP-evoked Ca^{2+} release via its target channels TPC1, TPC2and RyR1.

• We investigated the role of JPT2/HN1L in NAADP-dependent Ca^{2+} signaling across four distinct cell types.

• NAADP-dependent Ca^{2+} responses were unaltered in *JPT2/HN1L*$^{-/-}$ cardiomyocytes, platelets, and mast cells.

• JPT2/HN1L is essential for NAADP-mediated $CD4^{+}$ T-cell receptor activation.

Abstract

Nicotinic acid adenine dinucleotide phosphate (NAADP) is a second messenger evoking Ca^{2+} release from intracellular Ca^{2+} stores by targeting several Ca^{2+} channels including two-pore channels (TPC1/2), transient receptor potential mucolipin-1 (TRPML1), or ryanodine receptor type 1 (RYR1). For activation of Ca^{2+} channels, NAADP requires binding proteins, such as JPT2/HN1L and LSM12. So far, their function has been analyzed in several cell lines and only

a very limited number of primary cells; however, their physiological relevance in cell types known to utilize NAADP signaling remains unclear. Here, we generated JPT2/HN1L-deficient (*Jpt2/Hn1l*$^{-/-}$) mice to evaluate the contribution of JPT2/HN1L proteins to platelet aggregation, and Ca^{2+} signaling in cardiomyocytes, mast cells, and $CD4^{+}$ T cells. NAADP is known to contribute to collagen-related peptide (CRP)-evoked platelet aggregation, but this was not altered by JPT2/HN1L deletion. Functional Ca^{2+} imaging revealed that JPT2/HN1L plays a strikingly cell-type specific role in NAADP-mediated Ca^{2+} release. In beating ventricular cardiomyocytes, β-adrenergic stimulation is known to evoke arrhythmogenic spontaneous diastolic Ca^{2+} transients, which were not altered in their frequency in *Jpt2/Hn1l*$^{-/-}$ myocytes. Further, antigen-evoked Ca^{2+} transients in peritoneal mast cells (PMCs) are not changed in *Jpt2/Hn1l*$^{-/-}$ PMCs. However, $CD4^{+}$ T cells displayed a pronounced requirement for JPT2/HN1L. Following T cell receptor/CD3 stimulation, global Ca^{2+} elevations and early NAADP-driven Ca^{2+} microdomains, which occur within tens of milliseconds of TCR/CD3 engagement and serve as initiating signals for downstream immune activation, were significantly decreased in *Jpt2/Hn1l*$^{-/-}$ $CD4^{+}$ cells. We conclude that JPT2/HN1L is indispensable for NAADP-mediated Ca^{2+} release in T cells, but dispensable in cardiomyocytes, platelets, and mast cells, at least for the agonists employed. Accordingly, LSM12 might compensate for the loss of JPT2/HN1L. Together, JPT2/HN1L is not universally required as an NAADP-binding protein but exhibits cell-type specificity, with an essential function in T cell Ca^{2+} signaling.

Introduction

Nicotinic acid adenine dinucleotide phosphate (NAADP) was first described in 1995 as a highly potent Ca^{2+} releasing second messenger in sea urchin egg homogenate. Afterwards, NAADP was also shown to evoke Ca^{2+} release in several eukaryotic cell types, demonstrating its universal role as a Ca^{2+} mobilizing second messenger. NAADP has been characterized as a potent Ca^{2+} mobilizing agent in mammalian cells, influencing Ca^{2+} release from intracellular stores and playing pivotal roles in cellular signaling across diverse cell types (Churamani et al. 2004; Ruas et al. 2015).

Although NAADP has been identified as a universal Ca^{2+}-mobilizing second messenger, its function exhibits specificity within distinct cell types, including T cells (Berg et al. 2000; Dammermann et al. 2009), neurons (Hermann et al. 2020), mast cells (Arlt et al. 2020), cardiomyocytes (Capel and Terrar 2015), and platelets (Rosado 2011). In T cells, NAADP signaling contributes to the formation of Ca^{2+} microdomains that are crucial for initiating immune responses (Gu et al. 2021). In cardiomyocytes, β-adrenergic stimulation leads to Ca^{2+} release from endo-lysosomes originating from activation of two-pore channels (TPC2) and followed by amplification via CaMKII and RYR2 at lysosomal/sarcoplasmic reticulum (Lyso-SR) junctions (Zong et al. 2009). This Ca^{2+} release evokes spontaneous diastolic Ca^{2+} transients (SCTs) in cardiomyocytes as the initial event triggering arrhythmias under catecholaminergic stress (Capel et al. 2015; Nebel et al. 2013). In platelets, NAADP has been reported to release Ca^{2+} from acidic Ca^{2+} stores (e.g., SERCA3-dependent stores) within seconds upon stimulation, thereby mediating platelet activation and aggregation (Coxon et al. 2012; Feng et al. 2020).

The targets of NAADP-induced Ca^{2+} signaling comprise a range of endo-lysosomal and sarcoplasmic/endoplasmic reticulum (ER) channels, most notably two-pore channels (TPC1 and TPC2), transient receptor potential mucolipin-1 (TRPML1), and ryanodine receptor type 1 (RYR1). TPC channels are structurally unique dimers with six transmembrane domains, are expressed at diverse positions within the endo-lysosomal continuum, and act as the primary NAADP-sensitive channels (Lin-Moshier et al. 2014; Morgan and Galione 2014). The functional significance of TPCs in NAADP signaling is underscored by their role in modulating physiological processes that vary across cell types. TPC1 is typically associated with early endosomes and plays a role in endosomal recycling, whereas TPC2 predominantly localizes to lysosomes and is integral to lysosomal Ca^{2+} signaling and endocytic trafficking (Castonguay et al. 2017; Morgan et al. 2011). Knockout models for TPC1 and TPC2 have elucidated several roles in cellular processes, including arrhythmogenesis, as well as cardiac remodeling under catecholamine stress (Capel

et al. 2015; Nebel et al. 2013) or the development of cardiac dysfunction after ischemia/reperfusion (Davidson et al. 2015). In mast cells, TPC1 deficiency or blockade augments systemic anaphylaxis and mast cell activity (Arlt et al. 2020).

Even before the discovery of TPCs as NAADP-sensitive Ca^{2+} channels, RYR1 was proposed to be activated by NAADP (Hohenegger et al. 2002) In fact, RYR1's open probability was increased by NAADP in lipid planar bilayer experiments using highly purified RYR1 in the presence of recombinant HN1L/JPT2 (Krukenberg et al. 2024). Upon TCR/CD3 stimulation, NAADP is formed in Jurkat T-lymphocytes (Gasser, Bruhn, and Guse 2006). Further, in $RYR1^{-/-}$ T cells NAADP initiated Ca^{2+} microdomains were significantly decreased (Diercks et al. 2018) . The involvement of RYR1 in NAADP-mediated Ca^{2+} signaling was also demonstrated by a decrease of NAADP-evoked Ca^{2+} signals upon antagonizing NAADP (Dammermann et al. 2009) or upon blocking the RYR1 with antibodies (Dammermann et al. 2009; Gerasimenko et al. 2015). Data on pharmacological inhibition of RYR1, gene silencing and RYR1 knockout T cells have shown that RYR1 is responsible for the formation of initial, short-lived Ca^{2+} microdomains in T cells. These rapid local Ca^{2+} signals within the first 15 seconds upon T cell activation have been characterized as being NAADP-dependent (Guse and Wolf 2016; Wolf et al. 2015). The NAADP-evoked Ca^{2+} rise is absent in MEF cells from *Trpml1*$^{-/-}$ mice, indicating that TRPML1 channels are a third potential NAADP target (F. Zhang et al. 2011).

A fundamental aspect of NAADP signaling is the requirement for NAADP-binding proteins, as TPC channels are not necessary for NAADP binding (Ruas et al. 2015), and NAADP has never been shown to bind directly to RYR1 or TRPML1. Recently, two key proteins, JPT2/HN1L and LSM12, have been proposed as NAADP-binding proteins that act to facilitate NAADP's interaction with these channels. JPT2/HN1L was identified via NAADP photoaffinity labeling, protein purification and mass spectrometry as a potential NAADP-binding protein, and its knockout in Jurkat T lymphoma cells and primary rat effector T cells results in attenuated Ca^{2+} signals following TCR/CD3 stimulation (Gunaratne et al. 2021; Roggenkamp et al. 2021); in parallel, JPT2/HN1L was also identified in additional cell lines as NAADP binding protein (Gunaratne et al., 2021)) (Gunaratne et al. 2021). LSM12, discovered through proteomic approaches as a binding partner of both TPC2 and NAADP, has been shown to have a similar capacity to modulate NAADP-dependent Ca^{2+} release, with reduced Ca^{2+} signaling observed in LSM12-deficient cells (J. Zhang et al. 2021). Despite this progress, the roles of these binding proteins in specific cellular contexts of NAADP signaling remain to be elucidated. In this study, we generated JPT2/HN1L-deficient (*Jpt2/Hn1l*$^{-/-}$) mice, which enabled us to compare the role of JPT2/HN1L in four primary cell types, for which NAADP signaling has been reported to be triggered after stimulation with

(patho)physiological relevant agonists, i.e. platelets, ventricular cardiomyocytes, mast cells and CD4+ T cells from both *Jpt2/Hn1l*$^{-/-}$ mice and littermate controls. Our results show that JPT2/HN1L deletion reduces TCR/CD3 mediated global Ca^{2+} elevations and early NAADP-driven Ca^{2+} microdomains. In contrast, NAADP-dependent Ca^{2+} signaling in ventricular myocytes and mast cells, as well as platelet aggregation, were unaltered. These results thus show that the relevance of JPT2/HN1L is cell-type dependent and suggest a complementary role for other NAADP-binding proteins, possibly LSM12.

Results

Generation of *Jpt2/Hn1l*$^{-/-}$ mice

To investigate the role of JPT2/HN1L in primary cell types, we used a genome editing approach in mouse zygotes using two guide RNAs flanking exon 3 (Fig1. A), to generate founder mice as described(Medert et al. 2023). Founder animals harboring a *Jpt2/Hn1l* null (-) allele with deletion of exon 3 (143bp, detected by PCR, Fig1. B), resulting in a premature stop codon, were bred with C57Bl6/N mice. The obtained heterozygous *Jpt2/Hn1l*$^{+/-}$ offspring were intercrossed to *Jpt2/Hn1l*$^{-/-}$ mice (Suppl. Fig. 1A-B). *Jpt2/Hn1l*$^{-/-}$ are viable and fertile. Lack of JPT2/HN1L protein expression in the spleen of *Jpt*2/Hn1l$^{-/-}$ mice was confirmed by Western blot analysis (Fig. 1C).

JPT2/HN1L does not play a role in catecholamine-induced Ca^{2+} release in cardiomyocytes

To test whether JPT2/HN1L plays a role in Ca^{2+} signaling in electrically evoked Ca^{2+} transients, we measured the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) in freshly isolated ventricular cardiomyocytes. Electrical pacing evoked 1.17±0,06 (mean±SEM) spontaneous diastolic Ca^{2+} transients (SCT) in *Jpt2/Hn1l*$^{+/+}$ cells and 1.14±0.08 SCT in Jpt2/Hn1l$^{-/-}$ cardiomyocytes (Fig. 2A). Furthermore, amplitude of Ca^{2+} transients was comparable between genotypes, where *Jpt2/Hn1l*$^{+/+}$ cells presented with an amplitude of 0.39±0.05 and *Jpt2/Hn1l*$^{-/-}$ cells with 0.47±0.06 upon electrical stimulation.

Next, we tested whether JPT2/HN1L contributes to NAADP-mediated Ca^{2+} release in primary ventricular cardiomyocytes. Catecholamine stimulation of beta-adrenergic receptors has been reported to generate NAADP in cardiomyocytes (Lin et al. 2017). In ventricular cardiomyocytes, NAADP targets TPC1 and TPC2, thereby inducing Ca^{2+} release from acidic Ca^{2+} stores, such as endosomes and lysosomes. Therefore, we performed Ca^{2+} imaging using the β1-adrenoceptor agonist isoproterenol (ISO). In cardiomyocytes treated with 100 nM ISO, Ca^{2+} amplitudes increased to 0.46±0.11 and 0.66±0.07 in *Jpt2/Hn1l*$^{+/+}$ and *Jpt2/Hn1l*$^{-/-}$, respectively. Furthermore, we observed an increase in the number of SCTs in *Jpt2/Hn1l*$^{+/+}$ cardiomyocytes of 2.06±0.13 peaks per stimulus and similarly 2.00±0.19 peaks per stimulus in *Jpt2/Hn1l*$^{-/-}$ cells (Fig. 2B, Supple. Fig. 2). Further increasing the concentration of ISO to 300nM resulted also in similar number of SCT (Fig. 2C) in Jpt2/Hn1l$^{+/+}$ (2.50±0.02 peaks per stimulus) compared to *Jpt2/Hn1l*$^{-/-}$ (2.29±0.15 peaks per stimulus) and an amplitude in intracellular Ca^{2+} of 0.35±0.04 and 0.39±0.13 in *Jpt2/Hn1l*$^{+/+}$ and *Jpt2/Hn1l*$^{-/-}$ cardiomyocytes, respectively (Fig. 2C, Suppl. Fig. 2). 3-Isobutyl-1-methylxanthin (IBMX) is a phosphodiesterase (PDE) inhibitor. PDEs catalyze the degradation of

cAMP and can be inhibited to prolong its action. cAMP has been suggested to induce Ca^{2+} release which is reduced in *TPC2*$^{-/-}$ cells (Chang et al. 2020). We aimed to further enhance the ISO-evoked Ca^{2+} effect by combining ISO with IBMX. Nevertheless, no change in frequency (Jpt2/Hn1l$^{+/+}$: 2.20±0.08, Jpt2/Hn1l$^{-/-}$: 2.57±0.39 SCTs) or amplitude (*Jpt2/Hn1l*$^{+/+}$: 0.46±0.11, *Jpt2/Hn1l*$^{-/-}$: 0.48±0.34) was observed between genotypes (Fig. 2D, Suppl. Fig. 2).

NAADP-mediated platelet aggregation is unaffected in *Jpt2/Hn1l*$^{-/-}$ thrombocytes

Upon activation, platelets undergo morphological changes and form aggregates. This activation is tightly regulated by signaling molecules, particularly cytosolic Ca^{2+} levels (Rosado 2011). Stimulation of Collagen-Related Peptide (CRP) activates the GP-VI receptor, which has also been postulated to evoke NAADP-dependent Ca^{2+} release and aggregation (Coxon et al. 2012). We stimulated thrombocytes with CRP at a concentration that induced aggregation, and this aggregation was prevented by pre-incubation with the NAADP inhibitor Ned-19. However, platelet aggregation upon stimulation of 0.3µg/ml CRP was unchanged in JPT2/HN1L-deficient platelets (Max: 77.9±0.5%, AUC: 43.7±2.8) compared to litter controls (Max: 80.8±6.0%, AUC: 43.8±5.2) (Fig. 3 A). Taken together, these data show that loss of JPT2/HN1L does not alter NAADP-mediated aggregation in mouse platelets.

In Addition to GPVI receptors, PAR4 activation has also been described to generate NAADP in thrombocytes. PAR-4 receptors can be stimulated by low concentrations of thrombin (0,1 U/ml) as well as by PAR-4-specific agonists, e.g., y-thrombin or PAR-4A peptides (Jardin et al. 2007; Soslau et al. 2004). For these agonists, we performed dose-response curves to determine IC50-values for aggregation (Suppl. Fig. 3).

However, aggregation upon stimulation of the PAR-4 receptor using 0.1 U/ml thrombin resulted in a maximal aggregation of 77.9±4.6% and 80.2±3.8% and an AUC of 53.3±2.9 and 54.2±4.2 of Jpt2/Hn1l$^{+/+}$ and Jpt2/Hn1l$^{-/-}$ (Fig. 3 B), respectively. We also observed no significant difference in aggregation upon stimulation with the PAR4 specific agonist γ-thrombin which resulted in a maximal aggregation of 75.4±6.2% and an AUC of 36.7±5.5 in Jpt2/Hn1l$^{+/+}$ cells and a maximal aggregation 88.7±1.5% of and an AUC of 51.4±2.5 in Jpt2/Hn1l$^{-/-}$ platelets (Fig. 3C). Similarly, PAR4-A, another agonist specifically for PAR4 receptor, evoked an aggregation in Jpt2/Hn1l$^{+/+}$ thrombocytes (Max: 79.1±4.7%, AUC: 44.9±4.6) which was indistinguishable from Jpt2/Hn1l$^{-/-}$ platelets (Max: 87.6±1.4%, AUC: 53.1±1.2) (Fig. 3D).

Peritoneal mast cells

In peritoneal mast cells (PMCs), treatment with the NAADP antagonist Ned-19 increased the antigen (DNP)-evoked intracellular Ca^{2+} rise, similar to deletion of TPC1 channels, suggesting that NAADP-mediated activation of TPC1 counteracts antigen-evoked Ca^{2+} rise (Arlt et al. 2020). To test the impact of the deletion of the NAADP-binding protein JPT2/HN1L in the antigen-evoked Ca^{2+} signaling in PMCs, we sensitized PMCs with anti-IgE antibodies directed against DNP. We studied the DNP-evoked changes in $[Ca^{2+}]_i$ in PMCs from *Jpt2/Hn1l*$^{-/-}$ and litter-matched *Jpt2/Hn1l*$^{+/+}$ controls. The Ca^{2+} rise evoked by antigen stimulation (DNP 10 ng/ml) did not differ in amplitude or AUC between genotypes (Fig. 4 A-F).

JPT2/HN1L is essential for NAADP-dependent Ca^{2+} microdomains in $CD4^+$ T cells

NAADP serves as a pivotal second messenger in the initial Ca^{2+} signaling events that begin within seconds of TCR/CD3 engagement. Basal Ca^{2+} levels in *Jpt2/Hn1l*$^{-/-}$ T cells were unchanged compared to *Jpt2/Hn1l*$^{+/+}$ controls. However, upon stimulation of TCR by 1µg/ml anti-CD3, a significant reduction in Ca^{2+} peak was observed in JPT2/HN1L-deficient T cells compared to control cells (Fig. 5A). To analyze the NAADP-mediated Ca^{2+} release further, we investigated the earliest detectable Ca^{2+} signals, the Ca^{2+}microdomains that occur predominantly below the plasma membrane close to the immune synapse (Fig. 5B). These Ca^{2+} microdomains form within tens or hundreds of milliseconds of TCR/CD3 ligation and precede global Ca^{2+} increases. In *Jpt2/Hn1l*$^{-/-}$ T cells, a significant decrease of the number of Ca^{2+}microdomains was observed, both as integrated over the first 15s (Fig. 5C), or as kinetics data (Fig. 5D). Together, these data confirm the relevance of JPT2/HN1L in NAADP-mediated Ca^{2+} release in $CD4^+$ T cells.

Discussion

In this study, we generated a JPT2/HN1L knockout mouse and systematically assessed the involvement of JPT2/HN1L as an NAADP-binding protein in four primary cell types (cardiomyocytes, platelets, mast cells, and $CD4^+$ T cells). All these cell types have previously been described to utilize NAADP as a second messenger for Ca^{2+} mobilization from intracellular stores. Our findings have provided new insights into the physiological relevance of JPT2/HN1L, demonstrating apparent cell-type specificity in its role in NAADP-mediated Ca^{2+} signaling. JPT2/HN1L is not required for catecholamine-induced Ca^{2+} release in ventricular cardiomyocytes and for NAADP-dependent platelet aggregation. Similarly, NAADP-mediated Ca^{2+} release in mast cells upon DNP stimulation was unaffected by deletion of JPT2/HN1L. However, it was critical for the generation of early NAADP-driven Ca^{2+} microdomains in murine $CD4^+$ T-cells.

Previous studies have implicated NAADP signaling in myocardial Ca^{2+} regulation, particularly during β-adrenergic stress and arrhythmogenesis (Capel et al. 2015; Nebel et al. 2013). NAADP has been shown to stimulate Ca^{2+} release mediated by TPC2 channels at lysosomal-sarcoplasmic reticular junctions of cardiomyocytes. Our data, however, demonstrate that loss of JPT2/HN1L did not affect Ca^{2+} transients under basal pacing or following isoproterenol stimulation, even when PDE inhibition potentiated the response. The frequency and amplitude of spontaneous Ca^{2+} waves and secondary Ca^{2+} transients remained similar between *Jpt2/Hn1l*$^{+/+}$ and *Jpt2/Hn1l*$^{-/-}$ cardiomyocytes across multiple conditions. Platelets are activated upon increased Ca^{2+} signaling, and NAADP has been proposed to contribute to platelet activation downstream of PAR-4 and GPVI receptors (Coxon et al. 2012; Rosado 2011). Importantly, pharmacological inhibition of NAADP with Ned19 partially impairs collagen-related peptide–induced aggregation, supporting NAADP's role as an intracellular amplifier. Our results, however, show that platelet aggregation triggered by low-dose thrombin, γ-thrombin, PAR-4 peptide, or CRP was preserved in JPT2/HN1L-deficient platelets, with no significant differences compared to controls. Finally, DNP has been shown to induce NAADP-mediated Ca^{2+} release via TPCs in mast cells; however, these Ca^{2+} signals were unchanged by genetic deletion of JPT2/HN1L. These findings imply that JPT2/HN1L is not required for NAADP-mediated signaling in cardiomyocytes, platelets, and mast cells, at least after stimulation with the agonists used in this study.

This could be explained by cell-type–specific reliance on distinct NAADP-binding proteins, or by the deletion of JPT2/HN1L, which evokes redundant mechanisms. Previous studies have described another NAADP-binding protein, LSM12 (J. Zhang et al. 2021). LSM12 was shown in HEK cells to interact with NAADP, TPC1, and TPC2 and to be involved in NAADP-evoked TPC

activation and Ca^{2+} mobilization from acidic Ca^{2+} stores (J. Zhang et al. 2021). Furthermore, it has been shown that both JPT2/HN1L and LSM12 bind NAADP and that JPT2/HN1L, and LSM12, function as NAADP-binding proteins in U2OS cells. In these cells, genetic deletion of Lsm12 was insufficient to suppress NAADP-mediated Ca^{2+} release completely. It required an additional knockout of JPT2/HN1L (Gunaratne et al., 2023), suggesting the possibility of redundant mechanisms between JPT2/HN1L and LSM12.

In contrast, JPT2/HN1L played a critical role in NAADP-mediated Ca^{2+} signaling in $CD4^+$ T cells. Of note, in contrast to cardiomyocytes and mast cells, NAADP triggers Ca^{2+} release in T cells via RYR1. Following TCR/CD3 stimulation, global Ca^{2+} elevations were significantly decreased in *Jpt2/Hn1l*$^{-/-}$ T cells. More markedly, the formation of Ca^{2+} microdomains immediately after TCR/CD3 ligation is a hallmark of NAADP-dependent signaling and was strongly reduced in JPT2/HN1L-deficient T cells. These highly localized Ca^{2+} transients serve as critical initiating events for T cell activation (Dammermann et al. 2009; Wolf et al. 2015), leading to downstream global Ca^{2+} signaling, NFAT activation, and gene transcription. Also, in Neuro2a cells JPT2 is indispensable for NAADP-evoked Ca^{2+} release and cannot be compensated by other NAADP binding proteins (Krukenberg et al. 2024).

Our data confirm previous evidence (Wolf et al., 2015, Diercks et al., 2018, Roggenkamp et al., 2021) (Diercks et al. 2018; Roggenkamp et al. 2021; Wolf et al. 2015) that JPT2/HN1L is critical for linking NAADP to RYR1-mediated Ca^{2+} release in T cells. Of note, this interaction of RYR1, JPT2/HN1L and NAADP was directly shown by increased RYR1 open probability when highly purified RYR1 was fused into lipid bilayers, and upon addition of NAADP and recombinantly expressed and purified JPT2/HN1L (Krukenberg et al. 2024). This suggests that the T cell signaling architecture differs from that of other studied cell types. indicating that the NAADP-binding proteins have cell-type-specific functions. The essential role of JPT2/HN1L in T cell Ca^{2+} signaling also positions it as a potential therapeutic target. Given the importance of early Ca^{2+} events in TCR signaling, modulation of JPT2/HN1L could represent a means of fine-tuning immune activation in settings such as autoimmunity, transplantation, or immunotherapy (Chen et al. 2024; Heßling, Troost-Kind, and Weiß 2023).

Together, these findings demonstrate that JPT2/HN1L is not universally required as an NAADP-binding protein but instead has cell-type–specific functions. In cardiomyocytes and platelets, JPT2/HN1L deficiency is well tolerated, suggesting that alternative NAADP-binding proteins (such as LSM12) or parallel Ca^{2+} release pathways can compensate. Conversely, T cells are critically dependent on JPT2/HN1L for the generation of early NAADP-driven Ca^{2+} microdomains,

highlighting a certain degree of specialization. Further studies should aim to dissect the interplay between JPT2/HN1L and LSM12, to uncover whether compensatory upregulation occurs in specific tissues, and to define the broader network of NAADP-interacting proteins. Importantly, our JPT2/HN1L knockout model provides a valuable tool to address these questions in vivo. Ultimately, delineating the mechanisms that confer cell-type specificity to NAADP signaling will be critical to harnessing this pathway for therapeutic interventions in cardiovascular disease, hemostasis, and immune regulation.

Materials and Methods

Animal Experiments

Mice were maintained under specified pathogen-free conditions in the animal facility (IBF) of the Heidelberg Medical Faculty. Mice were housed in a 12 h light–dark cycle, with a relative humidity between 56–60%, a 15-times air change per hour and room temperature of 22±2 °C. They were kept in conventional cages type II or type II long provided with animal bedding LTE E-001 (ABBEDD, Germany) and tissue papers as enrichment. Standard autoclaved food (Rod 16 or 18, Altromin, Lage, Germany) and autoclaved water were available to consume ad libitum.

Generation of the JPT2/HN1L knockout mouse:

To generate a JPT2 knockout mouse, we used a genome editing approach in mouse zygotes, which were injected with *heiCas9* mRNA (Thumberger et al. 2022) as described previously (Medert et al. 2023). We designed sgRNAs that induced double-strand breaks at the introns flanking exon 3 to cause deletion of the sequences between the sgRNAs, as shown previously (Medert et al. 2023) but without providing a donor sequence, following NHEJ-mediated repair. Deletion of exon 3 introduces a premature stop codon in the *Jpt2* coding sequence. Post-injection, embryos were transferred to pseudo-pregnant females, and founders were screened using a PCR genotyping strategy to confirm deletion. Six out of seven founder mice carried the null allele. Founder #6 was crossed to C57Bl6/N mice. Sequencing of DNA fragments amplified from genomic DNA of the *Jpt*2$^{+/-}$ offspring validated the absence of exon 3. Subsequently, all mice were genotyped by collecting ear punch biopsy at age of 3 weeks. Confirmation of genotypes was performed by PCR. *Jpt*2$^{+/-}$ mice were intercrossed to obtain *Jpt*2$^{-/-}$ offspring.

Western Blot

Mice were sacrificed and spleens were harvested and homogenised in lysis buffer containing protease inhibitors (1 mM Iodoacetamide; 1 mM Phenantholin; 0.1 mM PMSF; 1 µg/ml Antipain; 1 µg/ml Leupeptin; 0.7 µg/ml Pepstatin; 1 mM Benzamidine; 0.3 µM Aprotinin) using a tissue grinder (Tenbroeck Tissue Grinders, Wheaton. Protein concentration was determined by BCA assay. Immunoblotting of microsomal membrane fractions was performed as previously described (Medert et al. 2020). Samples were denatured at 60°C for 20 min and loaded Bis-Tris Plus gels, followed by gel electrophoresis at 150 V for 60 min. Resolved proteins were blotted onto

a 0.45 µm Protran nitrocellulose membrane (GE Healthcare) at 10 V for 60 min. Next, the membrane was blocked in 5% non-fat milk with TBS (pH 7.5). The Blot was incubated with an JPT2 (Sigma) or GAPDH (Acris) antibody at 4 °C overnight. Subsequently, the blot was washed three times for 5 min in TBST and incubated in horseradish peroxidase-conjugated anti-rabbit (GE Healthcare) and anti-mouse (Sigma) 1:50.000 at room temperature (RT) for 1 h. Afterwards, the blot was washed twice for 5 min in TBST and once for 5 min in TBS and then incubated with SignalFire Elite ECL (Cell Signalling) for 1 min. The chemiluminescence was detected by digital imaging (GE Healthcare, ImageQuant LAS 4000 mini). Original scans were saved as TIFF files from a LAS 4000 mini (GE Healthcare) and further analyzed using FIJI.

Platelet isolation and Aggregation

Blood sampling and animal handling were approved and performed according to the regulations of the Regierungspräsidium Karlsruhe and the University of Heidelberg conform to the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Blood was obtained by submandibular bleeding as described (Golde, Gollobin, and Rodriguez 2005) but using an 18G 11/2" needle under terminal Ketamine/Xylazine anesthesia (Xylazine-hydrochloride and Ketamine-hydrochloride final dose 16 and 120 mg/kg body weight, respectively; solved in sterile NaCl 0.9% and volume of 5 µL/g body weight; intraperitoneal injection). Washed platelets were isolated similarly as previously reported (Yang et al. 2022). Platelet numbers were determined and adjusted to 3×10^8 platelets/mL and cells were incubated for 30 min at 37 °C in the presence of 0.02 U/mL apyrase before aggregation experiments. Aggregation was determined at 37 °C using two APACT 4S Plus (Diasys, Holzheim, Germany) aggregometers. Tyrode's buffer served as reference value of 100% light transmission. A 190 µL platelet suspension was used and stimulated with corresponding agonists in a volume of 10 µL after 90 s of basal recording. In case of ADP-induced aggregation, measurements were done in the presence of 70 µg/mL fibrinogen (F3879, Sigma-Aldrich, Taufkirchen, Germany) added (4 µL) shortly before starting the measurements.

Isolation of adult mouse ventricular cardiomyocytes and intracellular Ca^{2+} measurements

For isolation of adult mouse ventricular cardiomyocytes, retrograde perfusion of coronary arteries was performed as described before (Camacho Londoño et al. 2015). Brief, the heart of an anaesthetized mouse was removed and cannulated from the aorta with a blunted 27G cannula

and fixed by a ligature. First, the heart was washed with an oxygenated EGTA containing (200 µM) Solution A+ for 3 min at RT. Subsequently, the heart was perfused with oxygenated digesting buffer (Solution A with 0.05 mg/ml liberase TM, Roche) for 2-3 min at 37°C until it became soft and pale. After digestion, the atria were separated from the ventricle. Ventricular cells were dissociated by gently pipetting, and undissociated tissue was removed by filtration through a 150 µm filter. The extracellular Ca^{2+} concentration was then stepwise increased to 1 mM. Cardiomyocytes were seeded on ECM-coated coverslips in Ca^{2+}-containing (1.8 mM) Tyrode's solution.

To investigate electrically evoked Ca^{2+} transients, Fura2-AM loaded cardiomyocytes were stimulated using an electrical field stimulator (MyoPacer Cell Stimulator, IonOptix) set to 5 V and 0.5 Hz and custom-made platin electrodes (Fachabteilung 3.1 Elektronik, Zentralbereich Neuenheimer Feld, Heidelberg University). Cells were continuously perfused with a Ca^{2+}-containing buffer throughout the measurement. The measurements were started without electrical field stimulation for 30 s. Afterwards, cells were stimulated with 5V at 0.5 Hz. After 5 min of measurement, cells were perfused with 100 nM ISO in 1.8 mM extracellular Ca^{2+} and the recording was paused for 5 min. Subsequently, the recording continued for an additional 5 min until the end of the measurement.

Peritoneal Mast Cell Isolation and intracellular Ca^{2+} measurements

Peritoneal mast cells (PMCs) were isolated and cultured as previously described (PMID: 30035759). Briefly, the cells were isolated by peritoneal lavage with RPMI medium, centrifuged (300 g) and subsequently re-suspended in 4 ml medium per mouse. The RPMI-based culture medium was supplemented with 20% fetal calf serum (FCS), 1% Penicillin-Streptomycin, 10 ng/mL of IL-3 and 30 ng/mL of Stem Cell Factor. The isolated cells were further cultured at 37 °C in 5% CO_2. Two days after isolation, the culture medium was replaced with fresh medium. On day nine, the cells were de-attached from the flask bottom by gentle pipetting and cultivated further at a concentration of 1×10^6 cells/mL in fresh medium in a new flask. The PMCs were used for the experiments on days 12-16 after isolation. For the experiments with antigen stimulation, the cells were treated overnight with 300 ng/mL anti-Dinitrophenyl IgE antibodies.

For Ca^{2+} imaging experiments, PMCs were loaded in a salt solution containing 2.5 µM Fura-2 AM and 0.1% Pluronic F-127 for 30 min at RT on the coverslips covered with concanavalin A (0.1 mg/mL). The intracellular free Ca^{2+} concentration was measured on the stage of AxioObserver-A3

inverted microscope (Zeiss, Germany) equipped with a 40x (1.3NA) immersion oil objective (Zeiss, Germany) in a perfused 0.5 mL chamber at RT. The Physiological Salt Solution (PSS) contained (in mM): NaCl 135, KCl 6, $CaCl_2$ 2, $MgCl_2$ 1.2, glucose 12 and HEPES 10; pH 7.4 (NaOH)). At the beginning of each experiment, the cells were washed thoroughly with PSS. The fluorescence signal was obtained by alternately exciting Fura-2 with 340 nm and 380 nm light (50 ms exposure time) using a pE-800fura (CoolLED, United Kingdom) light source. The emitted fluorescent signal was filtered for wavelengths>510 nm and detected by an AxioCam 503 mono CCD camera (Zeiss, Germany). The fluorescent ratio F340/F380 was measured with an acquisition rate of 5 s per cycle. Both, the camera and the Light source were controlled by the Zen 3.2 software (Zeiss, Germany).

Isolation of primary T-Cells and microdomain Ca^{2+} measurements

Fura2-AM, Fluo4-AM, and Fura-Red-AM were obtained from Life Technologies, dissolved in dimethyl sulfoxide and stored at −20°C. Anti-mouse CD3ε and anti-mouse CD28 mAb monoclonal antibodies were obtained from BD Biosciences. $CD4^+$ T-cells were isolated from spleens by negative selection using the EasySep Mouse $CD4^+$ T-Cell Enrichment Kit (STEMCELL Technologies Inc.) according to the manufacturer's instructions.

For global Ca^{2+} imaging, T-cells were loaded with 4µM Fura2-AM for 30 min at 37°C, washed twice in imaging buffer [140 mM NaCl, 5 mM KCl, 1 mM MgSO4, 1 mM $CaCl_2$, 20 mM Hepes (pH 7.4), 1 mM NaH_2PO_4, and 5 mM glucose] then kept at RT for 20 min for deesterification. Cells were placed on BSA (5 mg/mL) / Poly-L-Lysine (0.1 mg/mL) coated slides and stimulated with 1 µg/mL anti-CD3ε for 60s. 1.67 µM thapsigargin was added at 720 s as a positive control (data not shown). Images were acquired on a Leica IRBE microscope with 40-fold magnification, a Sutter DG-4 light source and an electron-multiplying charge-coupled device camera (Hamamatsu). One frame every 2 s was acquired using a Fura-2 filter set [excitation (ex), HC 340/26 nm, HC 387/11 nm; beam splitter (bs), 400DCLP; emission (em), 510/84 nm; AHF Analysentechnik]. Intracellular Ca^{2+} concentration was determined based on the equation of Grynkiewicz et al. 1985 (Grynkiewicz, Poenie, and Tsien 1985). Therefore, R_{min} [using the lowest ratio (R) and fluorescence (F) after EGTA chelation] and R_{max} (using the highest R and F after ionomycin incubation) of Fura-2 in single-cell measurements were assessed.

Imaging of Ca^{2+} microdomains in Fluo4 and FuraRed loaded T-cells was carried out as described in Gerlach et al. 2024 (Gerlach et al. 2024). Imaging was carried out with an exposure time of 25

ms (40 frames/s) in a 14-bit mode using a dual-view module (Optical Insights, PerkinElmer Inc.) to split the emission wavelengths [filters: ex, 480/40; bs, 495; emission 1 (em1) 1, 542/50; em2, 650/57]. The DARTS pipeline for postprocessing and analysis was used (Woelk et al. 2023).

Statistical analysis

Results were analyzed using OriginPro2019 (OriginLab) and Graphpad Prism 8.0. Values are shown as mean ± SEM. Statistical significances were assessed using the appropriate statistical tests (unpaired Student's t-test, Mann-Whitney U test or Two-Way ANOVA). Significance is depicted as * $p < 0.05$; ** $p < 0.01$, *** $p < 0.001$.

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Figures

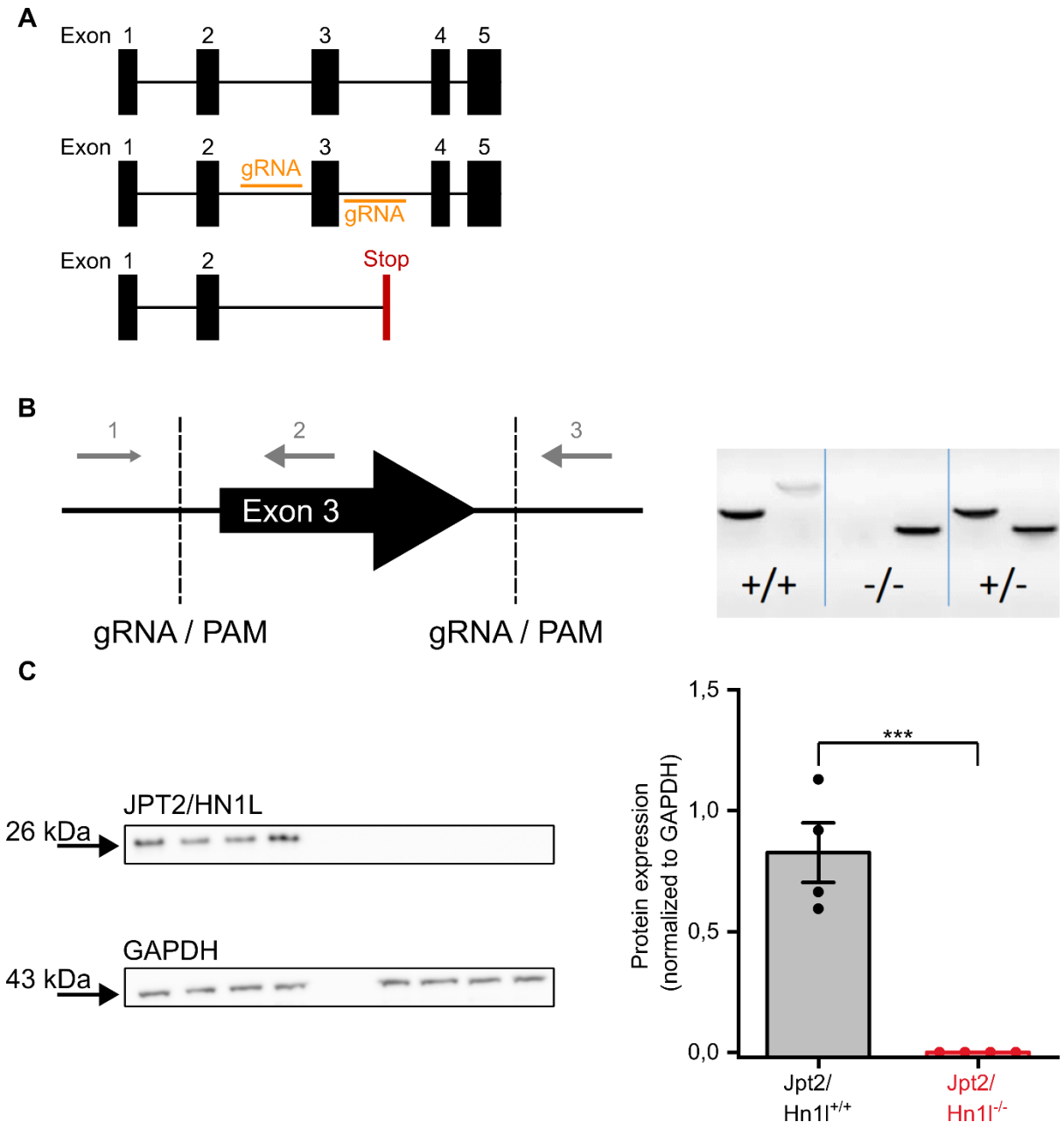

Figure 1: Generation of a *Jpt2/Hn1l* knockout Mouse. (A) cloning strategy of generation of *Jpt2/Hn1l* knockout mouse using CrisPR-Cas9. Using two gRNAs flanking exon 3 to delete exon 3 and cause a premature stop codon. **(B)** PCR to show genetic deletion of exon 3 by Q5-polymerase PCR using primers flanking exon 3. Left reaction (Primer 1 (*Jpt2*-WT/KO-fw) and 2 (*Jpt2*-WT-rev2)) results in WT band of 410bp) and right reaction (primers 1 (*Jpt2*-WT/KO–fw) and 3 (*Jpt2*-KO-rev)) shows a knockout band at 299bp and a faint WT band at 900bp. **(C)** Furthermore, deletion of JPT2/HN1L protein in spleens was confirmed by Western Blot using GAPDH as housekeeping protein. Each datapoint represents protein from a single mouse. Statistical analysis was performed according to unpaired Student's t-test, ***: $p < 0.001$.

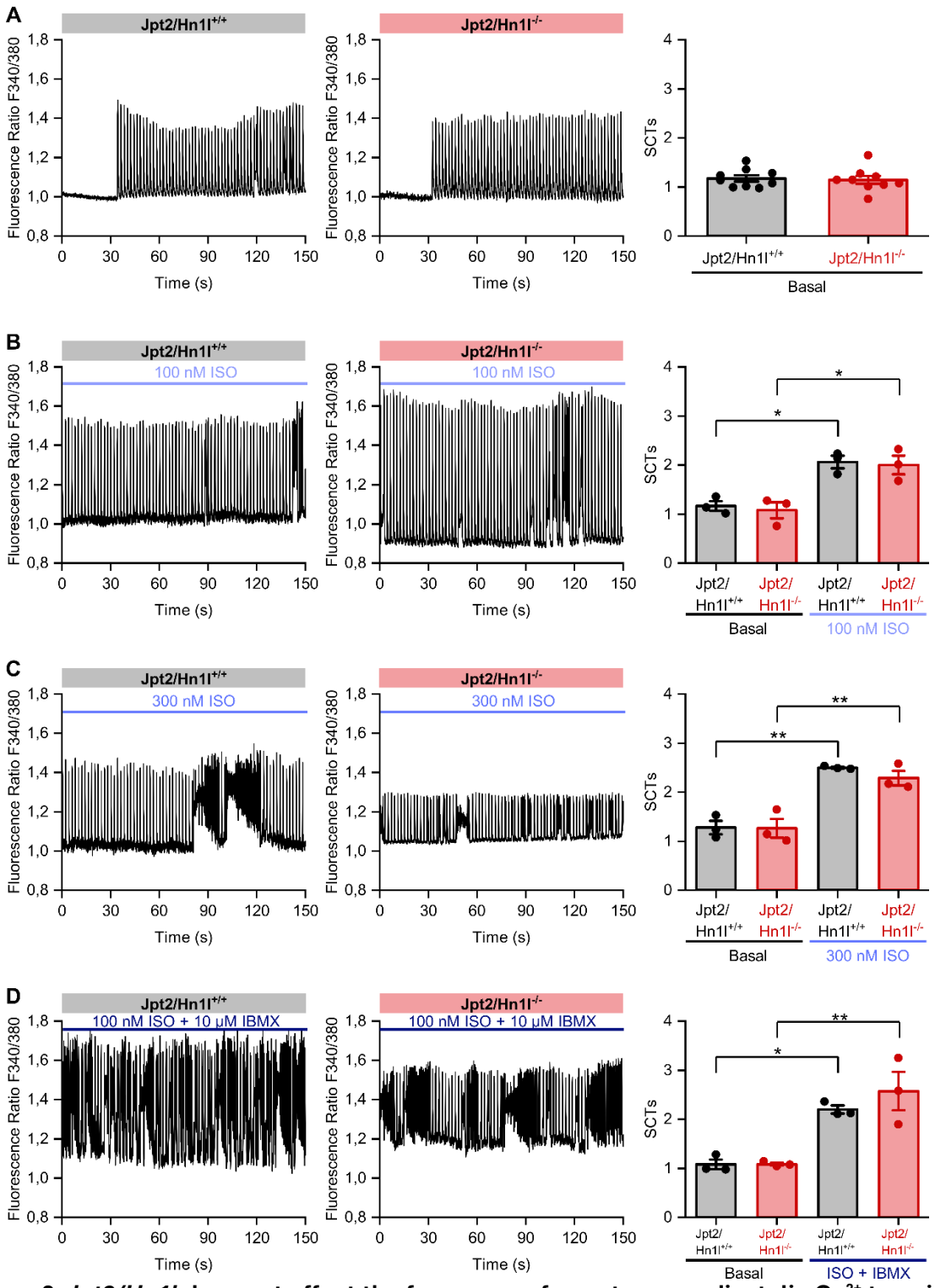

Figure 2: *Jpt2/Hn1l* does not affect the frequency of spontaneous diastolic Ca^{2+} transients (SCTs) in ventricular cardiomyocytes. (A) Pacing (0.5 Hz) ventricular cardiomyocytes with 20 Hz acquisition rate evokes Ca^{2+} signals which are independent of JPT/HN1L. **(B-C)** Application of **(B)** 100 nM or **(C)** 300 nM ISO evoked an increased number of spontaneous Ca^{2+} events. **(D)** Finally, quantification of arrhythmogenic SCTs evoked by combining 100 nM isoprenaline with the phosphodiesterase inhibitor IBMX. Each datapoint represents an average of multiple cardiomyocytes of a single mouse and statistical analysis was performed using a Two-Way ANOVA with Bonferroni Post-hoc testing, *: $p < 0.05$, **: $p < 0.01$.

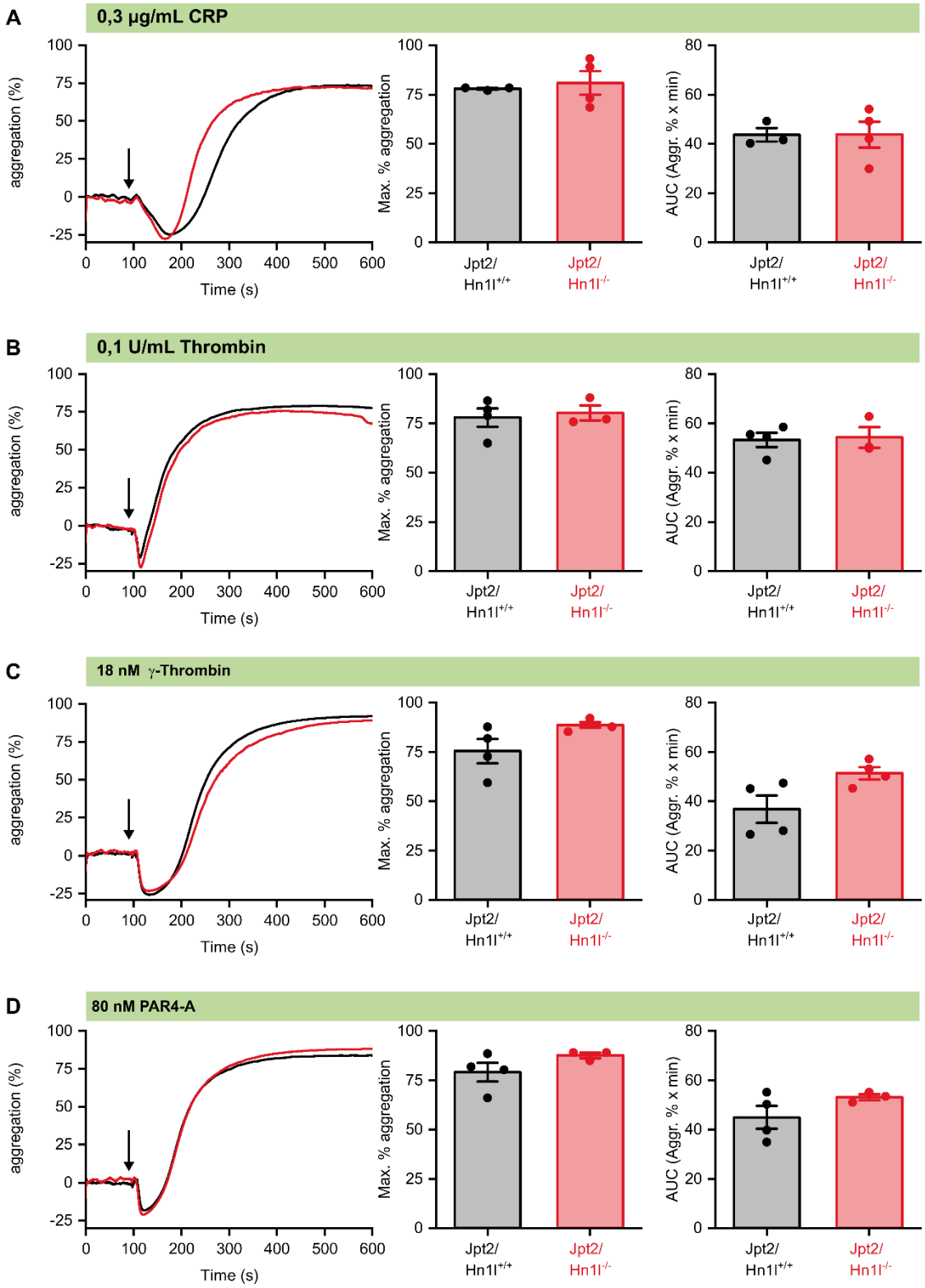

Figure 3: Platelet aggregation is independent of JPT2/HN1L. Platelet aggregation and quantification of maximal aggregation and AUC evoked by **(A)** CRP, **(B)** thrombin, **(C)** γ-thrombin or **(D)** PAR4-A in *Jpt2/Hn1l*$^{+/+}$ and *Jpt2/Hn1l*$^{-/-}$ platelets. Each data point represents a biological replicate of platelets isolated of a single group of mice. Statistical signifcance was tested using unpaired Student's t-test.

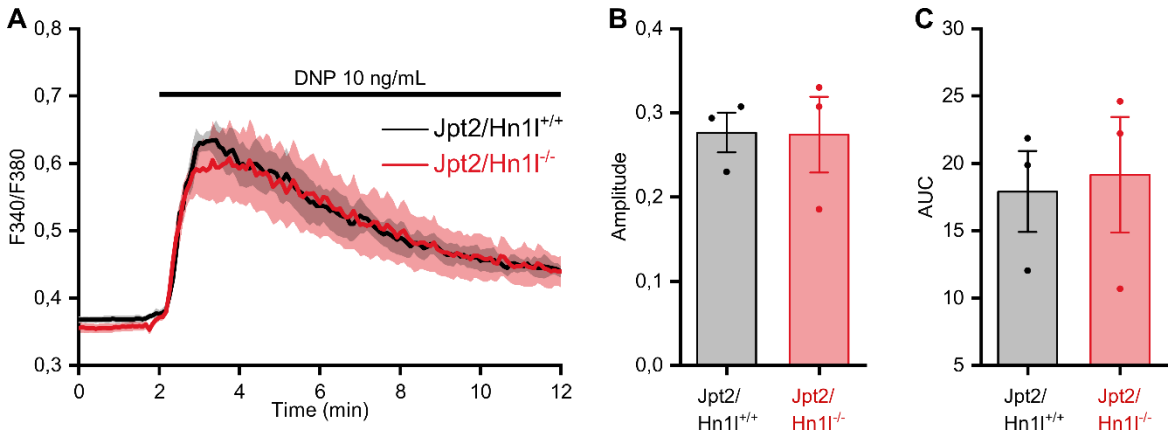

Figure 4: No difference in antigen-induced Ca^{2+} rise in *Jpt2/Hn1l*-deficient and wild type PMCs. (**A**) Mean traces (N=3) of changes of $[Ca^{2+}]_i$ shown as F340/F380 in PMCs isolated from *Jpt2/Hn1l*$^{-/-}$ (red) and litter-matched *Jpt2/Hn1l*$^{+/+}$ controls (black) stimulated with 10 ng/mL of antigen (DNP). **(B, C)** The corresponding statistical analysis of the amplitude (**B**) and area under the curve (**C**) in PMCs stimulated with 10 ng/mL of antigen and isolated from *Jpt2/Hn1l*$^{+/+}$ (black bars) and *Jpt2/Hn1l*$^{-/-}$ (red bars) mice (N=3 biological replicates).

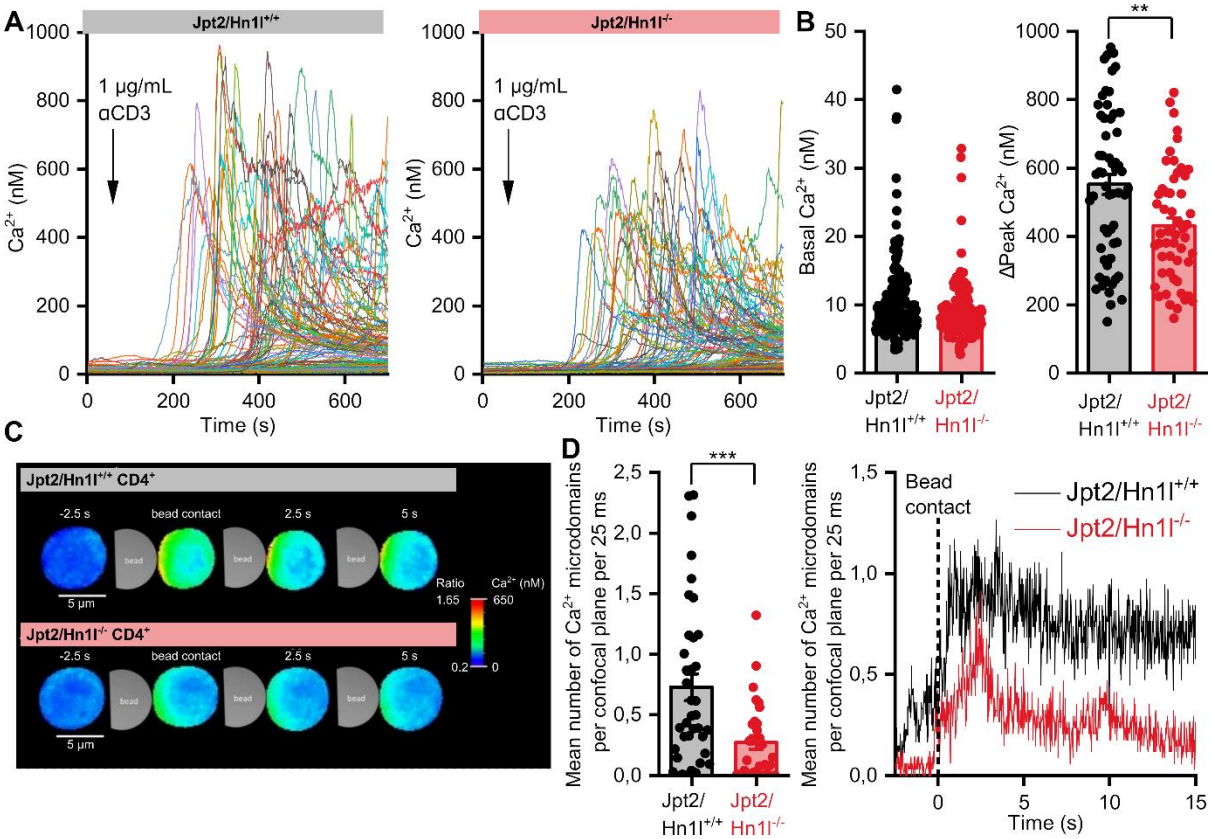

Figure 5: Global and local Ca^{2+} signals in CD4+ T-cells. (A, B) Analysis of global Ca^{2+} signals in $CD4^+$ T cells loaded with Fura2. **(A)** Whole cell Ca^{2+} traces after addition of 1 µg/mL soluble anti-CD3ε antibody at 60s. Images were acquired at 0.5 fps, n = 179 *Jpt2/Hn1l*$^{+/+}$ cells (left panel) and 150 *Jpt2/Hn1l*$^{-/-}$ cells (right panel) from N = 3 spleens per genotype. **(B)** Data points and average basal Ca^{2+} level and peak Ca^{2+} increase measured in Fura2-loaded T cells after anti-CD3ε treatment. 58 peaks detected in *Jpt2/Hn1l*$^{+/+}$ cells and 53 peaks detected in *Jpt2/Hn1l*$^{-/-}$ cells were compared with an unpaired t-test. **(C, D)** Analysis of Ca^{2+} microdomains in $CD4^+$ T cells. **(C)** Maximum intensity projections of 5 frames from representative cells showing Ca^{2+} microdomains at the bead contact site. The cells were loaded with Fluo4 and FuraRed and stimulated with anti-CD3ε/anti-CD28 beads for subcellular ratiometric Ca^{2+} imaging. Images were acquired at 40 fps. **(D)** Number of Ca^{2+} microdomains in 15s recording period (left) and average time course of Ca^{2+} microdomains before and after bead contact (right) from n = 37 *Jpt2/Hn1l*$^{+/+}$ and 31 *Jpt2/Hn1l*$^{-/-}$ cells derived from N = 6 spleens per genotype, statistical analysis with Mann-Whitney U test. **: $p < 0.01$, ***: $p < 0.001$.

Supplementary figures to:

JPT2/HN1L functions as an NAADP-binding protein in a cell-type specific manner

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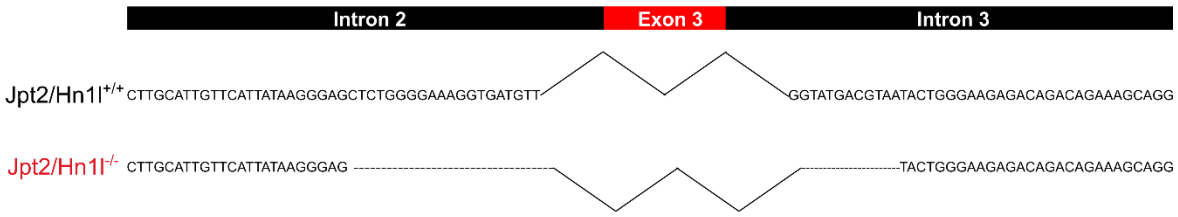
A



B

Complete deleted sequence - including Exon 3 in red

CTCTGGGGAAAGGTGATGTTTTTTTATTCCTTTTCTGAATCCAGACCCAGGTCAGCTTGGCTATATACACGT
GAACACTGTAGCAGACCAGATTAGAGATTAGCTGGGATATAAGGCTTCAGGGACTTGGAGTAATTGTCCCAC
TCTATTGTCCTGGAACCTAAGAAGGCATTAAGTCTTTTGTAGAAGGAAGACAGTGATGGCGTCGCATCAGAG
TTGAGCTCTGATGTTCACCCTTCTGTGATTGTTCCTGCAG**GAGGCAAAGGAAGTGGGATCTTTGATGAATC**
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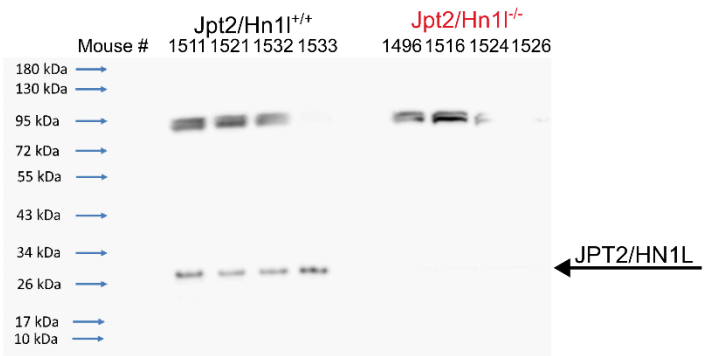
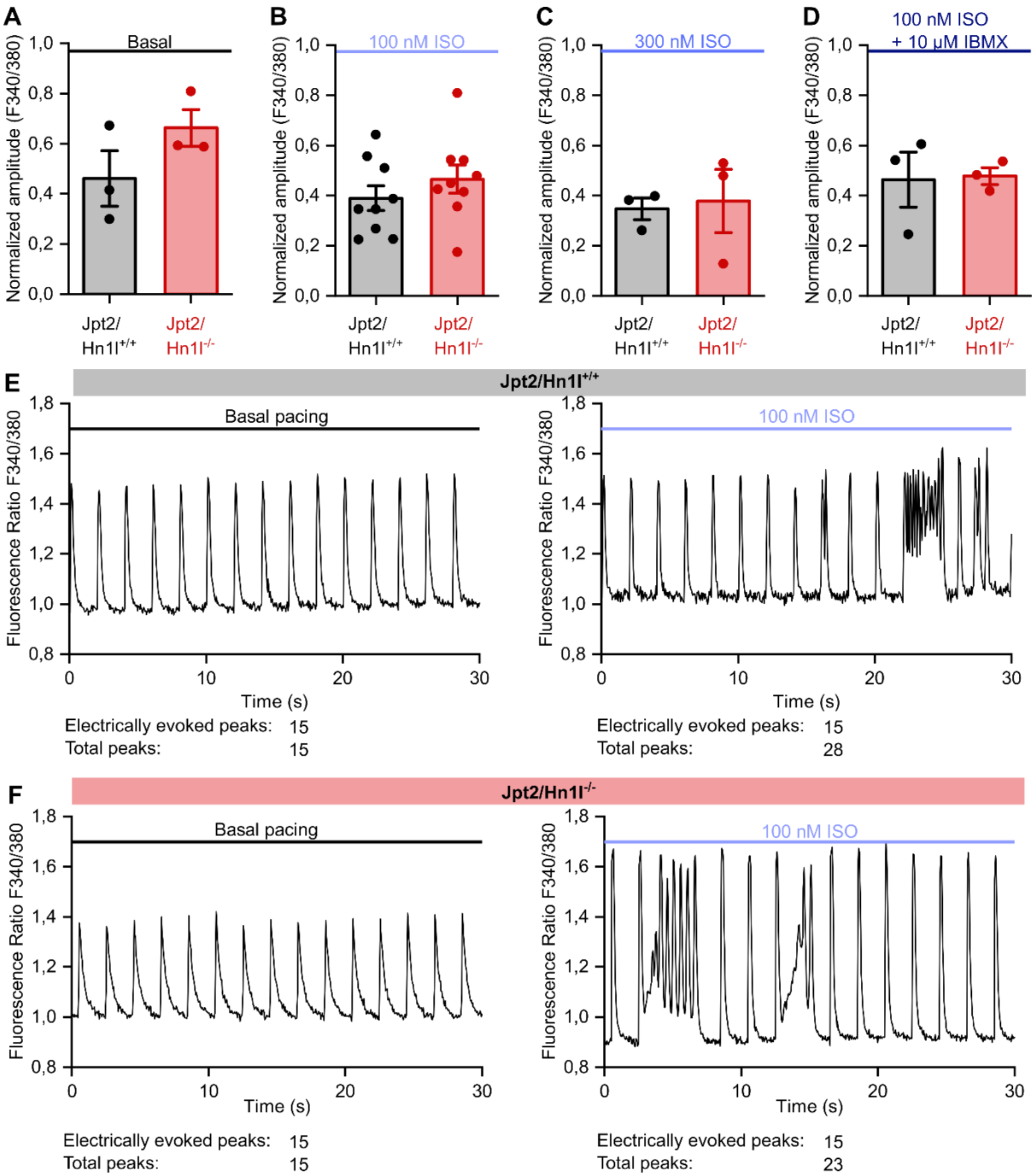
ATGACGTAA

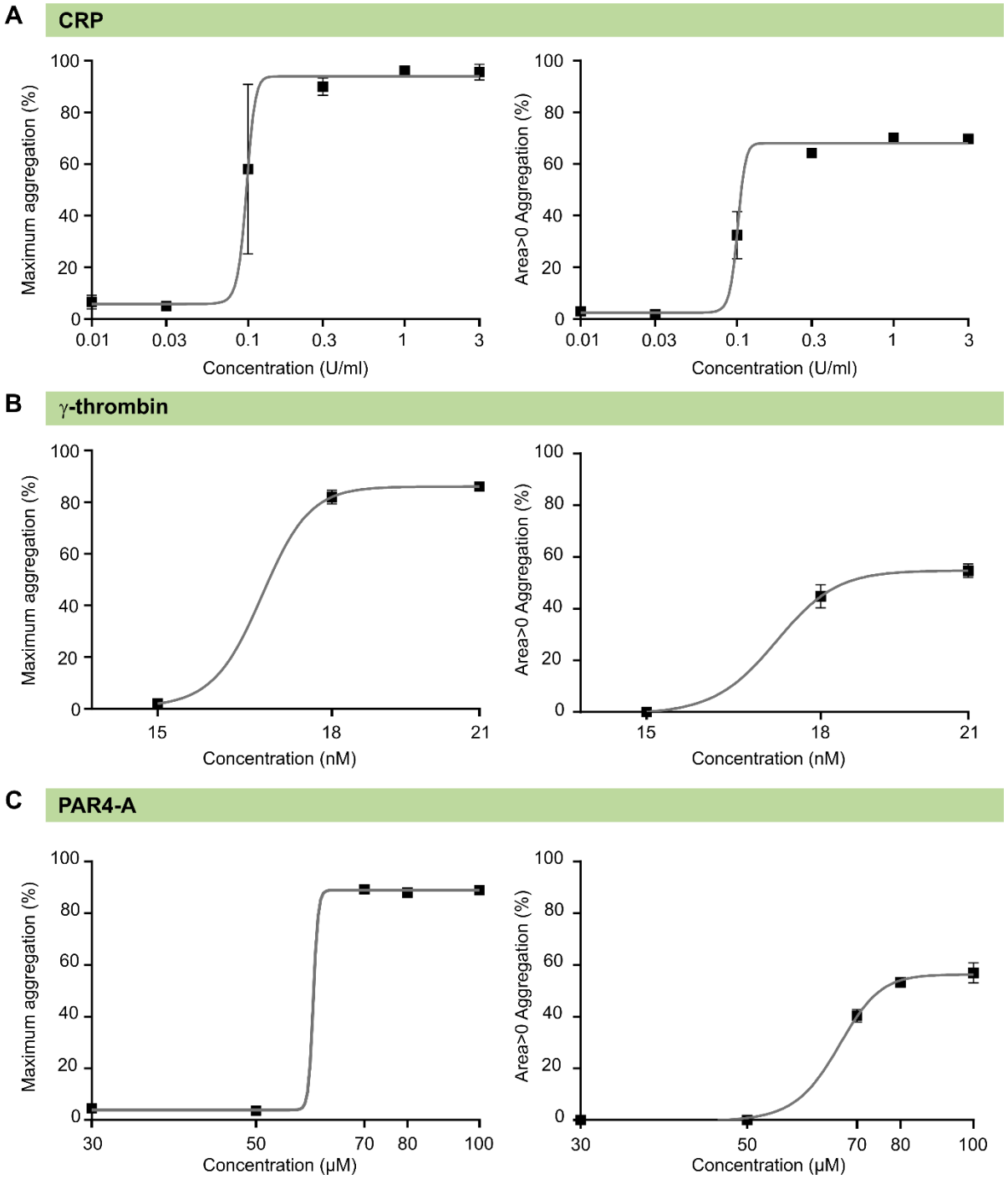
C



Suppl. Figure 1: Genetic deletion of *Jpt2/Hn1l* in mice. (A) We generated a *Jpt2/Hn1l* null allele by deletion of exon 3. **(B)** Complete sequence which was remove from the the genome was 577bp long, containing the 143bp long exon 3 (red). **(C)** We confirmed by Western Blot analysis that the 26/27 kDa protein is not expressed in our *Jpt2/Hn1l*$^{-/-}$ mice.



Suppl. Figure 2: Amplitude analysis of paced cardiomyocytes. Analysis of amplitude of Ca^{2+} transients evoked by **(A)** pacing alone or in combination with **(B)** 100 nM ISO, **(C)** 300 nM ISO or **(D)** a combination of 100 nM ISO and 10 µM IBMX. **(E)** A time segment with higher temporal resolution from the measurements in Figure 2A, B to illustrate the spontaneous Ca^{2+} transients evoked by 100 nM ISO in *Jpt2/Hn1l*$^{+/+}$ and **(F)** *Jpt2/Hn1l*$^{-/-}$ ventricular cardiomyocytes.



Suppl. Figure 3: Dose-response curve of aggregation of WT platelets. (A-C) Dose response curves of aggregation evoked by **(A)** CRP, **(B)** γ-thrombin and **(C)** PAR4-A peptide.