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

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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+} release channels, including two-pore channels (TPC1/2), transient receptor potential mucolipin-1 (TRPML1), or ryanodine receptor type 1 (RyR1). NAADP does not bind these channels directly but requires binding proteins, such as JPT2/HN1L and LSM12. So far, their function has been analyzed in several cell lines; 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 Ca2+ release. In beating ventricular cardiomyocytes, β-adrenergic stimulation is known to evoke arrhythmogenic spontaneous diastolic Ca2+ transients, which were not altered in their frequency in *Jpt2/Hn1l*$^{-/-}$ myocytes. 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 stimulation with anti-CD3, global Ca^{2+} elevations and early NAADP-driven Ca^{2+} microdomains, which occur within milliseconds of TCR 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, where the presence of LSM12 might compensate for the loss of JPT2/HN1L. Together, JPT2/HN1L is not universally required as an NAADP-binding protein but exhibits distinct 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 (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 (Calcraft et al. 2009; 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, while 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 the release of cytotoxic granules from NK-cells (Davis et al. 2012), and 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). Additionally, RyR1, traditionally associated with ER Ca^{2+} release, has been shown to mediate NAADP-evoked Ca^{2+} release in T-cells, where NAADP initiates microdomain Ca^{2+} signaling critical for early immune responses (Hermann et al. 2020; Wolf et al. 2015). Upon stimulation of the T-cell receptor on Jurkat T-lymphocytes, NAADP is formed, which activates the cells. The involvement of RyR1 in NAADP-mediated Ca^{2+} signaling has been demonstrated by a reduction of NAADP-evoked Ca^{2+} signals after 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 indicate 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 10 seconds after T-cell activation have been characterized as 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 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 and mass spectrometry as a potential NAADP-binding protein, and its knockout in T-cells results in attenuated Ca^{2+} signals following receptor stimulation (Gunaratne et al. 2021; Roggenkamp et al. 2021). LSM12, identified by proteomic approaches, has shown 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 T-Cells from both *Jpt2/Hn1l*$^{-/-}$ mice and littermate controls. Our results show that JPT2/HN1L deletion reduces T-cell receptor-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 the Ca^{2+} signaling in electrically evoked Ca^{2+} transients, we measured intracellular Ca^{2+} in freshly isolated ventricular cardiomyocytes. Electrical pacing evoked 1.17±0,06 (mean±SEM) spontaneous diastolic Ca2+ transients (SCT) in *Jpt2/Hn1l*$^{+/+}$ cells and 1.14±0.08 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 plays a role in NAADP-mediated Ca^{2+} release in primary ventricular cardiomyocytes. Catecholamine stimulation of the beta-adrenergic receptor has been described to generate NAADP in cardiomyocytes (Lin et al. 2017; Nebel et al. 2013) in ventricular cardiomyocytes NAADP targets TPC1 and TPC2, resulting in Ca^{2+} release from acidic Ca^{2+} stores, e.g. endosomes and lysosomes. Therefore, we performed Ca^{2+} imaging using the beta1-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, Suppl. 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 are responsible for the degradation of cAMP, and

by inhibiting cAMP-degradation. cAMP has been suggested to induce Ca^{2+} release which is reduced in *TPC2*$^{-/-}$ cells (Chang et al. 2020). We aimed to enhance the ISO-evoked Ca^{2+} effect further using a combination of ISO/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) is able to activate 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, which could be 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). Combined, these data show that loss of JPT2/HN1L does not change the 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$^{-/-}$, 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. 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).

Peritoneal mast cells

In peritoneal mast cells (PMCs), treatment with the NAADP antagonist Ned-19 increased the antigen (DNP)-evoked intracellular Ca2+ rise, similar to deletion of TPC1 channels, suggesting that NAADP-mediated activation of TPC1 counteracts antigen-evoked Ca2+ rise (Arlt et al. 2020). To test the impact of the deletion of the NAADP-binding protein JPT2/HN1L on the antigen-evoked Ca2+ signaling in PMCs, we sensitized PMCs with anti-IgE antibodies directed against DNP. We studied the DNP-evoked changes in intracellular Ca2+ concentrations in PMCs from Jpt2/Hn1l-/- and litter-matched Jpt2/Hn1l+/+ controls. The mean peak amplitude, mean peak width of DNP (1µg/ml) evoked Ca2+oscillations were not different between genotypes, nor were the fraction of oscillating cells nor the number of oscillations per cell. Larger and longer lasting Ca^{2+} transients evoked by a higher concentration of DNP (10µg/ml) did also not differ in amplitude or AUC between genotypes.

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 T-cell receptor (TCR) engagement. Basal Cal^{2+} levels in Jpt2/Hn1l$^{-/-}$ T-cells were unchanged compared to Jpt2/Hn1l$^{+/+}$ controls. However, upon stimulation of TCR by 1µg/ml αCD3, 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 calcium signals through localized microdomains. These microdomains form within milliseconds of TCR/CD3 ligation and precede global Ca^{2+} increases essential for T-cell function. We detected these Ca^{2+} microdomains in $CD4^+$ T-cells and detected 0,73 number of microdomains within the first 25ms after stimulation in Jpt2/Hn1l$^{+/+}$, which was significantly reduced to 0,27 Ca^{2+} microdomains in Jpt2/Hn1l$^{-/-}$ T-cells (Fig. 5B, C). Together, these data show the relevance of JPT2/HN1L in NAADP-mediated Ca^{2+} release in $CD4^+$ T-cells

Discussion

NAADP is a second messenger evoking Ca^{2+} release from intracellular Ca^{2+} stores but does not bind the channels which it activates (e.g., TPCs or RyR1) directly but requires binding proteins, such as JPT2/HN1L. The role of this protein has been investigated so far mainly in cell lines, and JPT2/HN1L is indispensable for NAADP-evoked Ca2+ release in Neuro2a cells (Krukenberg et al. 2024). Still, compensation by another NAADP binding protein, i.e. LSM12, was found in U2OS cells (Gunaratne et al. 2023). To study the causal contribution of JPT2/HN1L proteins in primary murine cells, we generated a JPT2/HN1L knockout mice. We assessed the role of JPT2/HN1L in four primary cell types, i.e. cardiomyocytes, platelets, mast cells, and $CD4^+$ T-cells, as all these cell types have previously been described to generate and 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 upon DNP stimulation of mast cells was unchanged 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 impact Ca^{2+} transients under basal pacing or following isoproterenol stimulation, even when potentiated by PDE inhibition. 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, but also 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 evoking 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 is involved in NAADP-evoked TPC activation and Ca^{2+} mobilization from acidic Ca^{2+} stores (J. Zhang et al. 2021). Furthermore, both JPT2/HN1L and LSM12 bind NAADP, and JPT2/HN1L, as well as LSM12, function as NAADP-binding proteins in U2OS cells. In these cells specifically, genetic deletion of Lsm12 was not sufficient to completely reduce NAADP-mediated Ca^{2+} release. It required additional knockout of JpPT2/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. Interestingly, in contrast to cardiomyocytes and mast cells, NAADP triggers Ca^{2+} release in T cells via RyR1. Following TCR stimulation, global Ca^{2+} elevations were significantly reduced in Jpt2/Hn1l$^{-/-}$ cells. More markedly, the formation of Ca^{2+} microdomains immediately after 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, leading to downstream global Ca^{2+} oscillations, NFAT activation, and gene transcription (Dammermann et al. 2009; Wolf et al. 2015) and similarly in rat T-cells and Jurkat cells (Roggenkamp et al. 2021). Also in Neuro2a cells (Krukenberg et al. 2024) JPT2 is indispensable for NAADP-evoked Ca^{2+} release and cannot be compensated by other NAADP binding proteins.

Our data provide evidence that JPT2/HN1L is critical for linking NAADP to RyR1-mediated Ca^{2+} release in T-cells. This suggests that T-cell signaling architectures rely on JPT2/HN1L in a way that other studied cell types do not, indicating that the NAADP-binding proteins have cell-type-specific functions. The essential role of JPT2/HN1L in immune 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 high 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 by PCR genotyping to confirm the 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 *Jpt2*$^{+/-}$ offspring validated the absence of exon 3. Subsequently, all mice were genotyped by collecting ear punch biopsy at age of 3 weeks. Genotype confirmation was performed by PCR. *Jpt2*$^{+/-}$ mice were intercrossed to obtain *Jpt2*$^{-/-}$ 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 a BCA Immunoblotting of microsomal membrane fractions was performed as described before(Medert et al. 2020). Samples were denatured at 37°C for 30 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 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, conforming 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 aggregometers (Diasys, Holzheim, Germany). Tyrode's buffer served as a 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 minutes at room temperature. 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 platinum 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 seconds. Afterwards, cells were stimulated with 5V at 0.5 Hz. After 5 minutes of measurement, cells were perfused with 100 nM ISO in 1.8 mM extracellular $Ca^{2+,}$ and the recording was paused for 5 minutes. Subsequently, the recording was continued for another 5 minutes 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 and 5% CO_2. Two days after the isolation, the culture medium was substituted with fresh medium. On day nine, the cells were detached 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 Ca2+ 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 room temperature on the coverslips covered with concanavalin A (0.1 mg/mL). The intracellular free Ca^{2+} concentration was measured on the stage of an AxioObserver-A3 inverted microscope (Zeiss, Germany) equipped with a 40x (1.3 NA) immersion oil objective (Zeiss, Germany) in a perfused 0.5 mL chamber at room temperature. 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 the Fura-2 with light of 340 nm and 380 nm wavelength (50 ms exposure time) using a pE-800fura (CoolLED, United Kingdom) light source. Emitted fluorescent signal was filtered at > 510 nm and detected by a charged-coupled device camera AxioCam 503 mono (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 room temperature 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 at 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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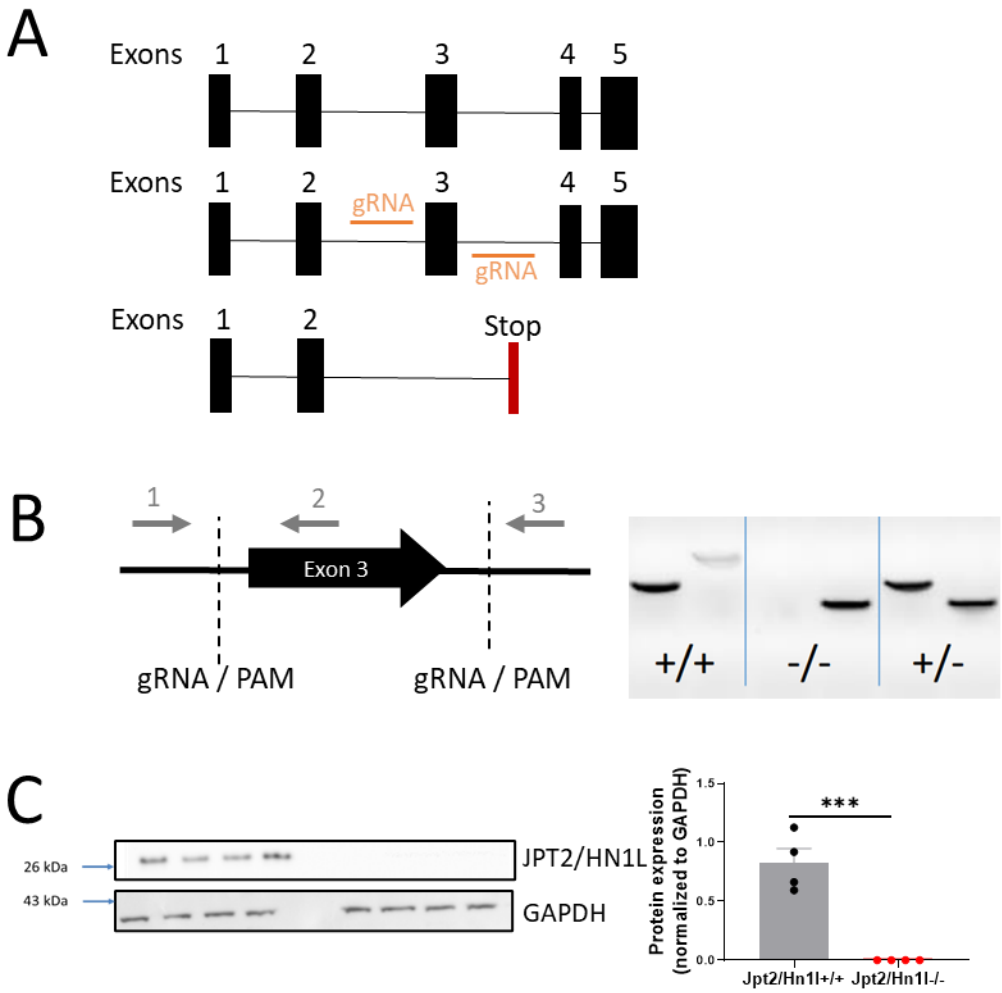

Figure 1: Generation of a Jpt2/Hn1l knockout Mouse. (A) cloning strategy of generation of Jpt2/Hn1l knockout mouse using CrisPR-Cas9. Using two gRNAs flanking exon 3 to delete exon 3 and cause a premature stop codon **(B)** PCR to show genetic deletion of exon 3 by Q5-polymerase PCR using primers flanking 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 protein in spleens was confirmed by Western Blot using GaPDH as house keeping protein. Each datapoint represents protein from a single mouse. Statistical analysis was performed according to unpaired Student's t-test.

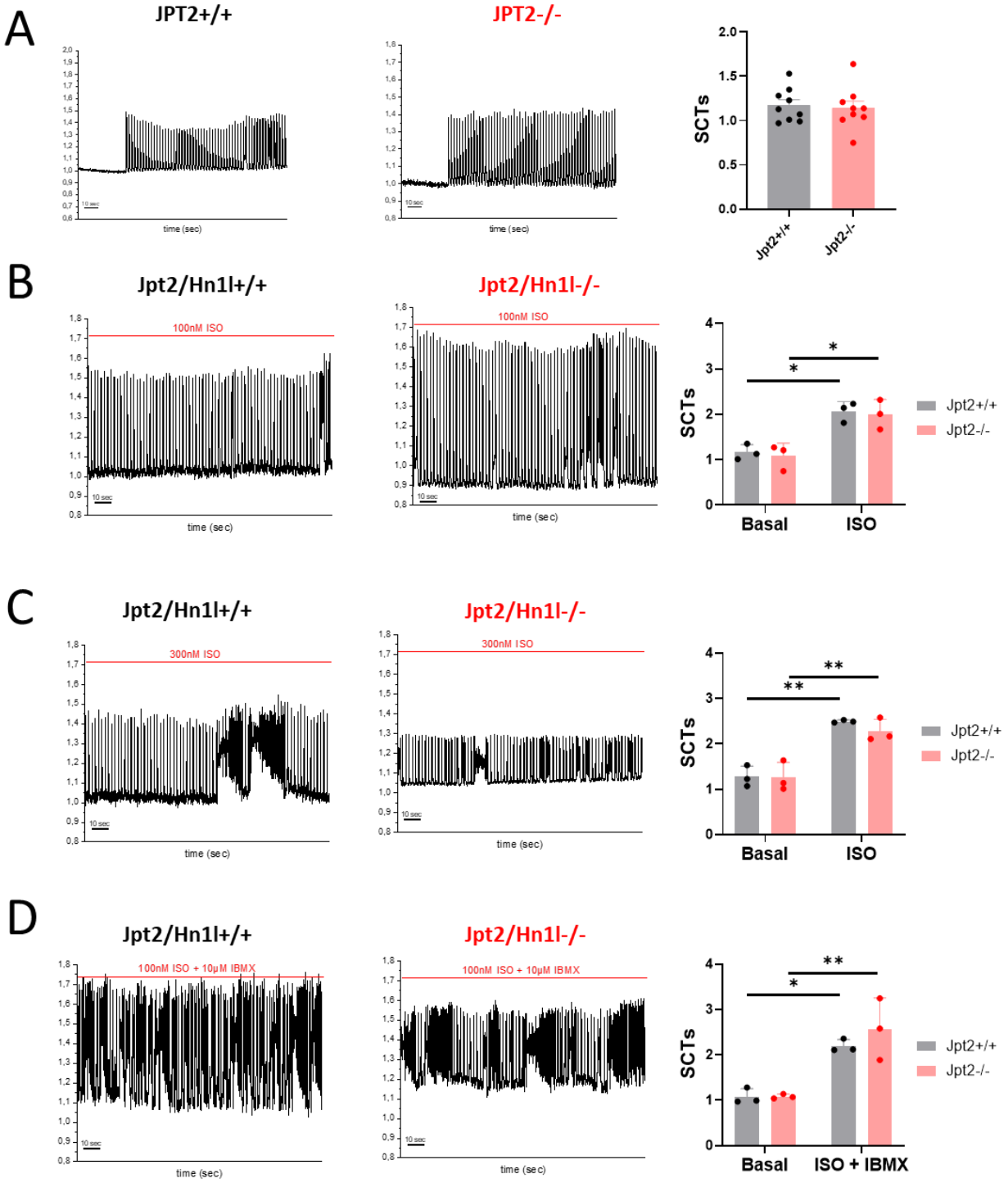

Figure 2: Jpt2/Hn1l does not affect the frequency of spontaneous diastolic Ca^{2+} transients (SCTs) in ventricular cardiomyocytes. (A) pacing (0.5 Hz) ventricular cardiomyocytes with 20Hz acquisition rate evokes Ca^{2+} signals which are independent of Jpt/Hn1l2. **(B-C)** Application of ISO **(B)** of 100nM or **(C)** 300nM evoked increased number of spontaneous Ca^{2+} events. **(D)** Finally, quantification of arrhythmogenic SCTs evoked by combining 100nM 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.

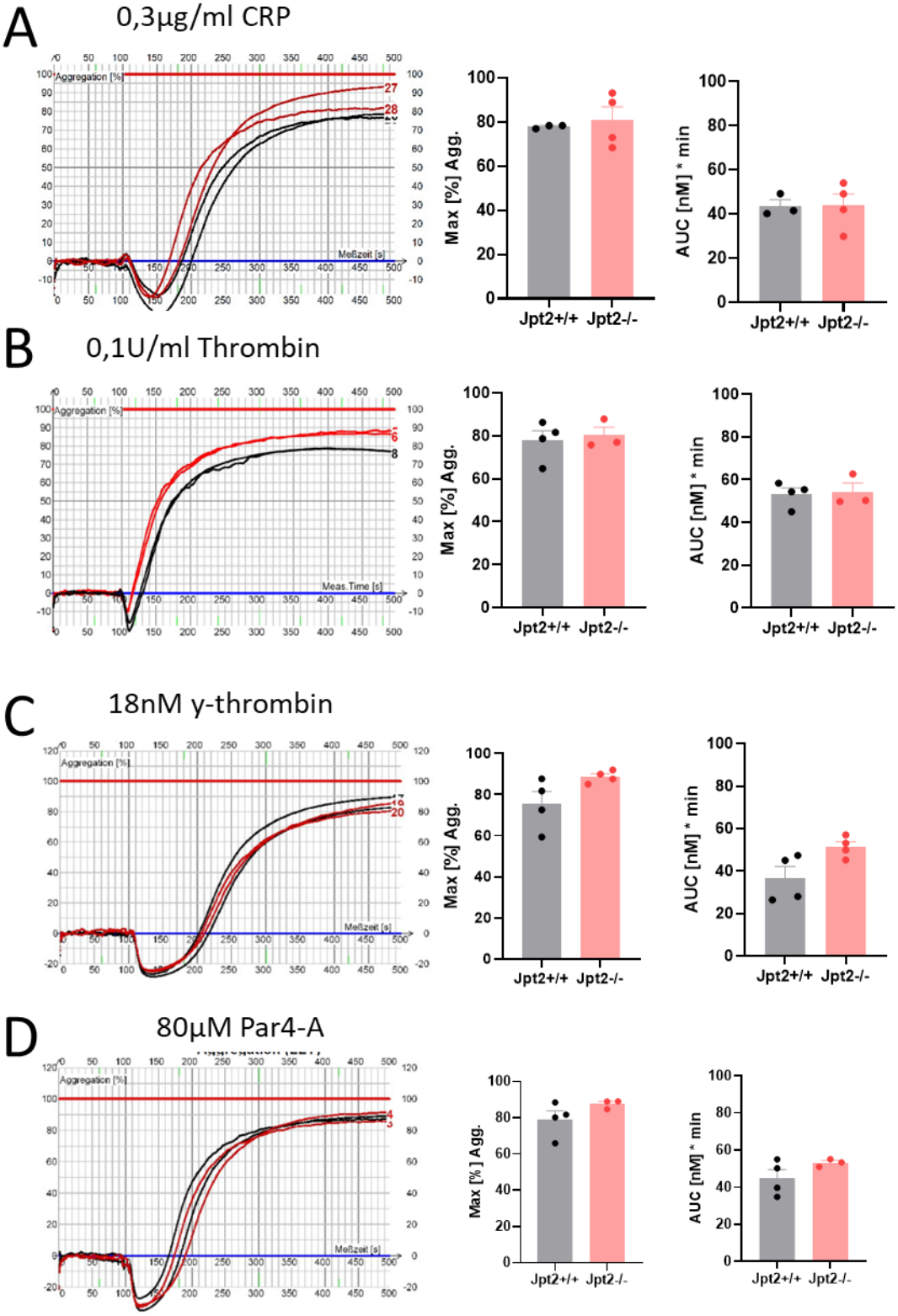

Figure 3: Platelet aggregation is independent of Jpt2/Hn1l. Platelet aggregation and quantification of maximal aggregation and AUC evoked by **(A)** CRP, **(B)** thrombin, **(C)** y-thrombin or **(D)** Par4-A in Jpt2/Hn1l$^{+/+}$ and Jpt2/Hn1l$^{-/-}$ platelets. Each data point represents a biological replicate of platelets isolated of a single group of mice. Statistical signifcance was tested using unpaired Student's t-test.

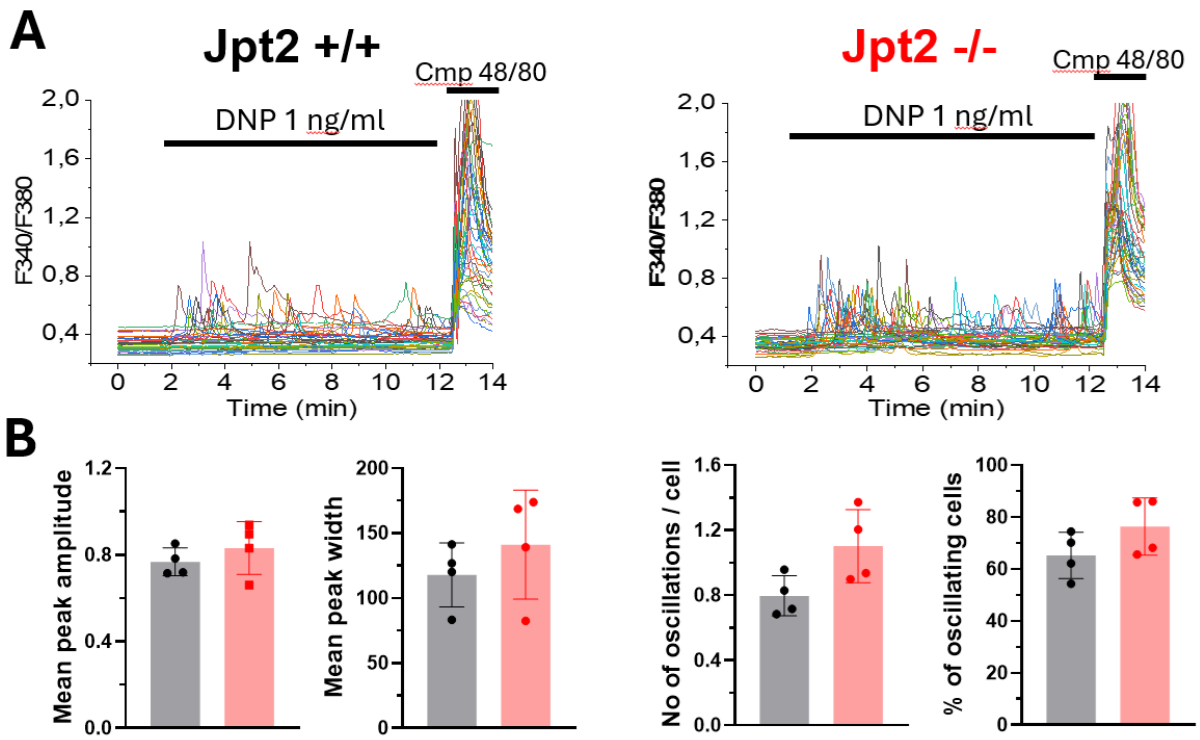

Figure 4. Antigen-induced Ca^{2+} oscillations in Jpt2-deficient and wild type PMCs. (**A**) Representative traces (n=40) of changes of $[Ca^{2+}]_i$ shown as F340/F380 in PMCs stimulated with 1 ng/ml of antigen (DNP) and 50 mg/ml of Compound 48/80 (Cmp 48/80) isolated from wild type (left) and Jpt2-deficient (right) mice. (**B**) The corresponding statistical analysis of the mean peak amplitude, mean peak width, number of oscillations per cell, percentage of oscillating cells in PMCs stimulated with 1 ng/ml of antigen and isolated from wild type (black bars) and Jpt2-deficient (red bars) mice (N=4). Statistical significance was tested according to unpaired Student's t-test

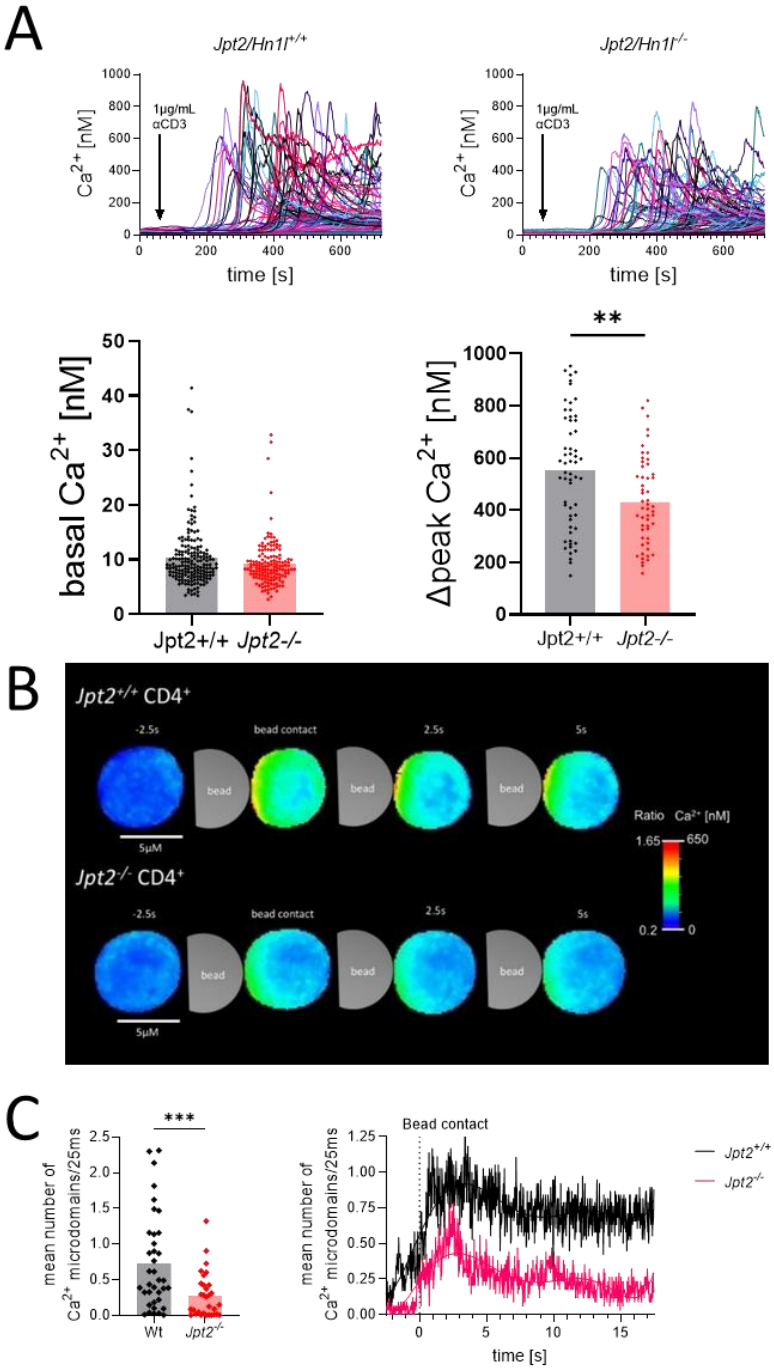

Figure 5: : Global and local Ca^{2+} signals in CD4+ T-cells. (A) Whole cell Ca^{2+} traces in $CD4^+$ T cells loaded with Fura2. Addition of 1µg/mL soluble anti-CD3ε antibody at 60s. Images were acquired at 0.5 fps, n = 179 wildtype and 150 knockout cells were analyzed from N = 3 spleens per genotype. **(B)** Basal Ca^{2+} level and peak Ca^{2+} increase measured in Fura2 loaded T cells after anti-CD3ε treatment. 58 peaks detected in wildtype cells and 53 peaks detected in knockout cells were compared by GraphPad Prism 10 unpaired t-test. **(C)** $CD4^+$ T cells 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, n = 37 wildtype and 31 knockout cells from N = 6 spleens per genotype. 5 frame maximum intensity projections of representative cells showing Ca^{2+} microdomains at the bead contact site. **(D)** Frequency and kinetics of Ca^{2+} microdomains after bead contact, statistical analysis with Mann-Whitney U test.