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Co-opting the unfolded protein response to elicit olfactory receptor feedback

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Summary

Olfactory receptor (OR) expression requires the transcriptional activation of one out of thousands of OR alleles and a feedback signal that preserves this transcriptional choice. The mechanism by which olfactory sensory neurons (OSNs) detect ORs to signal to the nucleus remains elusive. Here, we show that OR proteins generate this feedback by activating the unfolded protein response (UPR). OR expression induces Perk-mediated phosphorylation of the translation initiation factor eif2α causing selective translation of Activating Transcription Factor 5 (ATF5). ATF5 induces the transcription of Adenylyl Cyclase 3 (Adcy3), which relieves the UPR. Our data provide a novel role for the UPR in defining neuronal identity and cell fate commitment and support a two-step model for the feedback signal: first OR protein, as a stress stimulus, alters the translational landscape of the OSN and induces Adcy3 expression; then, Adcy3 relieves that stress, restores global translation and makes OR choice permanent.

Introduction

The mammalian main olfactory epithelium (MOE) is characterized by extreme diversity of olfactory sensory neurons (OSNs), each defined by the expression of a single olfactory receptor (OR) allele. In the mouse, the expressed OR is selected, in a monogenic, monoallelic and seemingly stochastic fashion (Chess et al., 1994) from a repertoire of more than a thousand genes (Buck and Axel, 1991). Heterochromatic silencing of all ORs, at a developmental stage that precedes their transcriptional activation (Magklara et al., 2011) and aggregation of the silent OR genes in distinct, heterochromatic nuclear foci (Clowney et al., 2012) assure their efficient repression and set the stage for the transcriptional activation of a single OR allele. Indeed, the active allele in each OSN is spatially separated from the repressed OR loci, interacts with the H enhancer, and carries activating histone marks (Clowney et al., 2012; Lomvardas et al., 2006; Magklara et al., 2011) suggesting that selective de-silencing of a single allele and relocation to a transcriptionally competent nuclear territory is the basis of OR activation (Magklara and Lomvardas, 2013). Lysine demethylase 1 (LSD1) plays a key role in this epigenetic switch, since it catalyzes the

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removal of repressive lysine 9 methyl marks from histone H3 on the chosen OR allele (Lyons et al., 2013). Importantly, the subsequent downregulation of LSD1 in response to OR expression prevents the de-silencing of additional ORs and stabilizes the expression of the activated allele revealing that LSD1 is the target of an OR-elicited feedback (Fleischmann et al., 2013; Lewcock and Reed, 2004; Nguyen et al., 2007; Serizawa et al., 2003) that locks OR choice for the life of the neuron (Lyons et al., 2013; Shykind et al., 2004).

The observation that the expression of OR protein causes the downregulation of LSD1 (Lyons et al., 2013) and, therefore, the stabilization of OR choice poses significant questions regarding the cellular mechanisms that elicit this feedback. OR gene activation induces expression of Adenylyl Cyclase 3 (Adcy3), which then signals for the downregulation of LSD1, providing a link between OR and LSD1 expression (Lyons et al., 2013). However, these results do not explain how an OR is detected by the neuron in the first place; Adcy3 plays a central role in the stabilization of OR choice, however, it is unlikely to be a “first responder” or initiator of the feedback, since its expression relies upon OR expression. Therefore, a central question towards the understanding of the OR feedback signal is how ORs are detected by the OSN and how this detection leads to the stable expression of Adcy3 protein. Because stabilization of OR choice requires the timely downregulation of LSD1 (Lyons et al., 2013) detecting and vetting the OR protein after targeting to the cell membrane may be too slow, since GPCR targeting requires an elaborate series of post-translational modifications and trafficking through the endoplasmatic reticulum (ER) and Golgi. Thus, protein quality control pathways placed in the first relay station of OR translation and processing, the ER, would rapidly link the onset of OR expression to Adcy3 transcription and, consequently, could provide a kinetic advantage for the stabilization of OR choice.

In the ER, a highly-conserved protein quality control pathway, the Unfolded Protein Response (UPR), acts to homeostatically adjust the ER environment upon detection of unfolded proteins. These adjustments include transcriptional induction of chaperones, acting to increase ER protein folding capacity, and inhibition of translation initiation, aiming to decrease ER load (Ron and Walter, 2007). The inhibition of translation initiation occurs downstream of the ER-resident kinase Perk, which in response to detection of unfolded proteins phosphorylates the translation initiation factor eif2α (Ron and Walter, 2007). This serves to limit the availability of tRNA^{met}, resulting in a general inability of ribosomes to initiate translation (Ron and Walter, 2007). Paradoxically, a small number of mostly stress-responsive mRNAs are preferentially translated under these conditions (Ron and Walter, 2007). This can be explained by the presence of inhibitory upstream open reading frames in their 5’-untranslated regions (5’-UTRs), which are selectively bypassed when tRNA^{met} becomes limiting, slowing ribosome assembly (Ron and Walter, 2007). Activating Transcription Factor 4 (ATF4), which is selectively translated under these conditions in many cell types, induces transcriptional changes that contribute to the clearance from the ER of misfolded proteins or to the adaptation of the ER to increased protein load (Harding et al., 2000a; Harding et al., 2000b).

Here, seeking to reveal the mechanistic outline of the OR feedback process, we test the hypothesis that UPR components detect OR proteins in the ER and transmit this information to the nucleus. Our experiments show that OR expression activates Perk in the neuronal ER, which phosphorylates the translation initiation factor eif2α, leading to selective and transient translation of Activating Transcription Factor 5 (ATF5), a paralogue to ATF4 that is highly transcribed in the MOE(Hansen et al., 2002). Translation of the nuclear form of Atf5 induces the transcription of Adcy3, which relieves the UPR, restores global translation, promotes OSN differentiation and stabilizes the expression of the chosen OR. PERK and ATF5 KO mice, as well as eif2α phosphorylation mutants, exhibit unstable OR expression

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and OSN maturation deficits, whereas pharmacological induction of the UPR or transgenic expression of nuclear ATF5 can bypass the lack of OR expression or the blockage of this signaling pathway. Our data solve a long lasting puzzle in OR regulation and provide a novel use for the UPR in neuronal differentiation and cell fate commitment that is likely applicable to other neurodevelopmental processes.

Results

ATF5 translation is regulated by OR expression

To test the idea that the UPR mediates the OR-elicited feedback signal we first examined the expression pattern of known components of this pathway in the nose. Our RNAseq analysis performed on sorted cell populations of the MOE (Colquitt et al., 2013; Magklara et al., 2011) corroborates reports that most components of the various UPR arms are highly transcribed in the MOE (Sammeta and McClintock, 2010; Sammeta et al., 2007). Intriguingly, unlike in most cell types, ATF5 expression is more than 100-fold higher than ATF4, making it one of the most highly expressed genes in mature OSNs (mOSNs) and progenitor cells (Globose Basal Cells, GBCs) (Figure 1A, and Hansen et al., 2002). However, immunofluorescence (IF) experiments on MOE sections with a highly specific antibody (Supplemental Figure S1A,B) reveal Atf5 protein expression only in immature OSNs, just prior to expression Adcy3, the major adenylyl cyclase that is expressed in response to OR expression and defines the mature OSN population (Figure 1B). The mutually exclusive expression pattern of nAtf5 and Adcy3 proteins contradicts the fact that at the mRNA level both genes are highly transcribed in mOSNs (Figure 1A). Because ATF5 mRNA is subject to alternative translation initiation (Watatani et al., 2008), we reasoned that the cause of the discrepancy between mRNA and protein expression of this gene relies on post-transcriptional regulation relevant to its biological function. Atf5, like its well-characterized paralogue ATF4, has upstream and downstream translation initiation sites that use different frames (Supplemental Figure S1D–G). The upstream ORF encodes for a small peptide, and the downstream ORF encodes for the nuclear transcription factor isoform (Zhou et al., 2008). Under physiological conditions only the upstream ORF is used, whereas upon ER stress and activation of the UPR, the downstream translation initiation site is preferentially used leading to the production of nuclear Atf5 (nATF5) (Watatani et al., 2008; Zhou et al., 2008), similarly with the established regulation of ATF4 (Harding et al., 2000a).

Because OR genes are among the most highly transcribed GPCRs, it is conceivable that the onset of their translation induces ER stress and activates the UPR, leading to both ER expansion that accommodates the increased protein load, and translational changes on ATF5. To test this idea, we performed IF for Atf5 in sections from LSD1 KO MOEs (FoxG1-Cre; $Lsd^{fl/fl}$), in which global OR expression is abolished (Lyons et al., 2013). These animals exhibit significant reduction of Atf5 immunoreactivity (Figure 1C,D), despite the fact that ATF5 mRNA remains highly abundant (Supplemental Figure S1C). Ectopic and mosaic expression of a transgenic OR, however, which partially rescues the differentiation deficits caused by LSD1 deletion (Lyons et al., 2013), restores Atf5 IF signal in the MOE (Figure 1D), suggesting that OR expression is required and sufficient for the translation of nATF5. Importantly, the cellular levels of the transgenic OR are lower than the cellular levels of the endogenous OR protein (data not shown) making unlikely that UPR induction is an overexpression artifact.

Atf5 Knockout Mice exhibit unstable OR expression

To test the potential role of ATF5 in the OR-elicited feedback, we obtained from KOMP an ATF5 KO mouse in which nAtf5 expression is abolished (Supplemental Figure S1A, B) and analyzed the effects of ATF5 deletion in OSN maturation and OR choice stabilization. In

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agreement with previous reports (Wang et al., 2012) we find a dramatic loss of mOSNs in Atf5 MOEs as assayed by Adcy3 IF at P0 (data not shown). A small number of ATF5 KO mice survive to adulthood, allowing a thorough characterization of the deficits caused by ATF5 deletion. Similarly with the newborn mice, we observe a dramatic loss of Adcy3 immunoreactivity in the ATF5 KO MOEs, with only a few persisting Adcy3-expressing OSNs with an intriguingly patterned spatial organization (Figure 2A, B); possibly these OSNs express a different class of chemoreceptors than the typical ORs, or they reflect the inefficient and infrequent rescue of ATF5 activity by ATF4. The widespread loss of Adcy3 expression in the ATF5 KO mice likely accounts for the sustained expression of LSD1 at the apical layers of the MOE (Figure 2C,D and Supplemental Figure S2A), in agreement with our previous findings (Lyons et al., 2013). Moreover, IF experiments with antibodies specific for OR proteins (Barnea et al., 2004; Lomvardas et al., 2006) show that in the ATF5 KO MOEs the numbers of OR-expressing neurons are significantly reduced and neurons that do express ORs have much weaker IF signal compared to control littermates (Figure 3E,F and Supplemental Figure S2B, C for quantitation for MOR28 IF signal and numbers of MOR28 expressing cells; similar results obtained for ORs M71 and M50, data not shown). RNA-seq analysis from control and ATF5 KO mice supports these observations showing significant reduction of OR mRNA in the ATF5 KO MOEs (Figure 2G). ATF5 deletion results in an even more dramatic downregulation of mature OSN marker expression (Figure 2H, Supplemental Figure S2C). Given that we already established that nAtf5 is produced only in response to OR expression, if this is the isoform involved in this process, then OR downregulation reflects OR choice stabilization defects and not deficiencies in initiation of OR expression, similar to the ones observed in the Adcy3 KO mice (Lyons et al., 2013). This hypothesis is supported by the sustained LSD1 expression in the ATF5 KO mice, which results in frequent OR gene switching.

To test this, we used a lineage tracing strategy that provides a reliable and reproducible readout for the stability of OR choice (Shykind et al., 2004). Briefly, we crossed the MOR28-IRES-Cre allele, which expresses Cre recombinase under the control of the highly-expressed OR MOR28, to the Cre inducible fluorescent reporter Rosa lox-stop-lox tomato (Madisen et al., 2010) and we counted the numbers of MOR28^{+}/tomato^{+} OSNs in control and ATF5 KO MOEs (schematic of this strategy in Figure 3A). Notably, due to incompatibility of Cre and tomato antibodies we can not distinguish between the Cre-expressing and the wild type MOR28 alleles, however the switching phenotype observed in the ATF5 KO is so robust (p-value <2.2e–16, Fisher's test) that this caveat does not affect the interpretation of this experiment. Specifically, in control animals, 68% of tomato-positive cells (n=199/293) continue to express MOR28 at 6 weeks of age, whereas, in ATF5 mutants only 11% of tomato-positive cells (n=27/234) continue to express MOR28 (Fig. 3B–C), supporting the notion that ATF5 KO OSNs undergo frequent OR switching.

Eif2α phosphorylation is required for nuclear ATF5 translation and OR Feedback

The demonstration that ATF5 is necessary for the stabilization of OR choice, together with the observation that translation of the nuclear ATF5 isoform is OR dependent, invite the hypothesis that the post-transcriptional regulation of ATF5 plays a crucial role on the feedback signal. As described above, the ATF5 transcript contains an inhibitory upstream open reading frame (iuORF), which is out-of-frame with the overlapping Atf5 coding sequence (CDS), such that the iuORF and CDS are translated in a mutually exclusive fashion (Watatani et al., 2008; Zhou et al., 2008) (Supplemental Figure S1D–G). Phosphorylation of the translation initiation factor eif2α relieves the translational inhibition imposed by the iuORF by slowing the assembly of translating ribosomes and allowing them to initiate translation at downstream ORFs, as seen with the regulation of ATF4 (Harding et al., 2000a).

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To test the role of eif2α phosphorylation in ATF5 translation in the MOE, we used a mouse line carrying a mutation in the phosphorylation site of eif2α (Scheuner et al., 2001). In these animals, eif2α delivers tRNA^{met} to assembling ribosomes even when eif2α kinases are activated, preventing stress-induced changes in translation initiation (Scheuner et al., 2001). Animals with this mutation die at birth, and as such Atf5 mRNA and protein expression were assayed only in newborns, where Adcy3 IF provides a robust and faithful proxy for the feedback signal (Lyons et al., 2013). In these mice we found that the Atf5 transcript is still abundant in RNA isolated from whole MOE (Supplemental Figure S3A). However, Atf5 protein is undetectable by IF in homozygote mutants (Figure 4A,B). Furthermore, these animals phenocopy Atf5 nulls, exhibiting a lack of Adcy3-expressing mOSNs, indicating a failure of ORs to elicit feedback (Figure 4A,B).

Because a number of other genes share the regulatory features described for Atf5, the loss of Adcy3 expression could be explained by loss of translation of mRNAs other than that encoding Atf5. Although analysis of the ATF4 KO mice did not reveal any OSN differentiation deficits or changes in gene expression (data not shown), we sought to further examine the potential contribution of additional genes in OR feedback with a genetic rescue experiment. We generated a tetO-regulated transgene encoding the nuclear isoform of Atf5 without its regulatory 5’-UTR, and forced its expression in immature OSNs with the use of the Ggamma8-tTA driver (Nguyen et al., 2007)(Figure 4C,D). Under this genetic strategy, nuclear ATF5 will be provided to the immature OSNs at a similar developmental stage with the endogenous nATF5, albeit with more sustained expression at the apical OSN layers (Figure 4D). Strikingly, expression of the nAtf5 transgene in the eif2α mutant background resulted in a rescue of Adcy3 transcription and translation (Figure 4C, Supplemental Figure S3C), suggesting that eif2a phosphorylation transmits the feedback signal predominantly through the transient translation of the nuclear ATF5 isoform. Notably, the rescue is not complete, since we detect fewer Adcy3 expression cell than in the wild type controls. However, this should be expected since the expression of transgenic nATF5 is not as efficiently regulated as that of the endogenous gene, which relies on tight translational control.

Unfolded protein stress in the ER elicits the OR feedback signal

Our experiments thus far propose that eif2α phosphorylation and translation of nuclear ATF5 are required for the induction of Adcy3 and the generation of the OR feedback signal. Since we already established that nATF5 is produced in response to OR expression we sought to identify the link between OR expression and eif2α phosphorylation, the ultimate molecular event before nATF5 translation. Phosphorylation of eif2α can be executed by at least four kinases (eif2αk1–4), each responsive to a distinct type of cellular stress (Ron and Walter, 2007). Among them, Perk (aka eif2αk3), which is activated upon detection of unfolded proteins in the ER lumen or in response to ER overload, presents the best candidate, because it would act as both a detector of ORs in the ER and transmitter of this information through eif2α phosphorylation.

To test involvement of Perk in the feedback pathway, we performed Atf5 IF in MOE sections of Perk KO mice (Harding et al., 2000). Perk KO mice, like eif2α phosphorylation mutants, die perinatally (Harding et al., 2000b), thus our analysis was also restricted to P0 mice. In these mice, we observe a complete loss of nAtf5 IF signal (Figure 5A,B, Supplemental Figure S4A), suggesting a dominant role of Perk in this pathway. Adcy3 expression is also abolished in the Perk KO MOEs (Figure 5A,B), further strengthening the major role of the UPR in the OR-elicited feedback pathway. Finally, expression of mature OSN markers is significantly reduced in the Perk KO, although expression of immature markers remains unaffected (Supplemental Figure S4A,B)

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This result invites the provocative hypothesis that at the onset of their translation ORs are viewed as unfolded proteins in the lumen of the ER, either directly by Perk or indirectly by other ER proteins. One prediction of this model is that pharmacologically inducing the UPR with tunicamycin, which activates the UPR by preventing protein N-glycosylation (Speake et al., 1980), should rescue OSN maturation in the absence of OR expression. To test this, we injected tunicamycin systemically into the LSD1 KO mice (LSD1fl/fl; FoxG1Cre), which do not express ORs (Lyons et al., 2013), and asked if we could induce nATF5 translation and OSN maturation in the complete absence of OR protein. Strikingly, tunicamycin injection rescued nAtf5 protein translation to wild-type levels (Figure 5C, Supplemental Figure S4A) and even induced Adcy3 expression in the cilia, where Adcy3 accumulates (Pace et al., 1985). However, Adcy3 protein expression did not reach wild type levels and only few neurons exhibited Adcy3 immunoreactivity in their cell bodies, suggesting that tunicamycin does not fully substitute for OR expression, which should be expected since it prevents translation initiation for most proteins. Thus, unfolded protein stress can substitute for OR expression to generate OR feedback; however, relief of this stress is also necessary for the fruition of this pathway and restoration of global OSN translation.

Adcy3 relieves the UPR to close the feedback loop

The fact that termination of the UPR is as crucial as the initiation of this pathway poses another puzzle in this unusual signaling mechanism; how is it that OR protein levels remain very high in the mature OSNs but the UPR, as visualized by nAtf5 protein expression, is only induced at the early stages of OR expression? Because we already established a crucial role of Adcy3 in this feedback process, an attractive model predicts that Adcy3 is involved in the termination of the signaling pathway that initiated its own transcription. To test this, we performed IF for Atf5 in MOE sections from Adcy3 KO mice, which have defects in OR choice stabilization (Lyons et al., 2013). In agreement with our hypothesis, in Adcy3 mutants, Atf5 immunoreactivity is greatly expanded (Figure 6A,B), despite a slight reduction of ATF5 mRNA (Figure 6C), supporting a role of Adcy3 in shutting off the UPR. Notably, in the Adcy3 KO mice LSD1 expression is sustained in the apical OSN layer resulting in frequent OR gene switching and reduced cellular levels of OR protein (Lyons et al., 2013). Therefore, the sustained UPR induction in the Adcy3 KO mice is not a consequence of elevated OR levels but likely caused by deficiencies in downstream molecular events evolved to relieve the OR-induced UPR. In any case, even if OR-induced ER stress is necessary for the generation of the feedback signal, relief of this stress via Adcy3 protein expression is equally important, since it restores general translation allowing terminal differentiation, LSD1 downregulation and the stabilization of OR choice. (Figure 7A,B)

Discussion

Our analysis suggests that OR expression in immature OSNs stimulates Perk, which phosphorylates eif2a leading to general stalling of translation initiation. Under these conditions, ATF5, one of the most highly expressed genes in the MOE, is translated from a downstream translation initiation site that produces a nuclear transcription factor isoform instead of the small peptide translated in the absence of ER stress. nATF5 activates the transcription of Adcy3 which eventually relieves the UPR, shuts off LSD1 expression, locks OR choice, and promotes terminal OSN differentiation (Figure 7A,B). Other limbs of the UPR pathway, i.e. Ire1 and ATF6 (Ron and Walter, 2007), are less likely to be involved in this feedback signal. Conditional deletion of Xbp1, the transcription factor produced Ire1, simultaneously with OR choice does not affect the stability of OR expression (data not

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shown), whereas ATF6 is expressed at low levels in the MOE, ~60fold lower than ATF5 based on our RNAseq analysis.

It is intriguing that instead of using ATF4, the canonical Perk-responsive transcription factor, this signaling process utilizes ATF5. Likely, the use of a paralogue with robust transcription in the MOE affords transcriptional responses tailored to the needs of this feedback, such as expression of Adcy3 and other OR signaling components. The fact that ATF4 KO mice do not have OR expression or OSN differentiation phenotypes (data not shown), together with the observation that ATF4 cannot compensate for ATF5 in the ATF5 KO, supports the notion that "hijacking" the UPR for OR feedback required the use of a specialized ER-stress induced transcription factor with different target specificity than ATF4.

An obvious question emerging from our studies regards the relief of the UPR in mOSNs, since both ORs and Perk remain highly expressed in these neurons. Perk activation may be prevented by OR-specific chaperones that inhibit Perk-OR interactions and/or clear the OR load from the ER by enhancing OR targeting to the cell membrane. Interestingly, RTP1 and RTP2, two ER-bound chaperones involved in OR targeting to the cell membrane (Saito et al., 2004), are expressed in the MOE in an ATF5-dependent manner (Supplemental Figure S2D). Thus, the expression of chaperones that could reduce the OR load from the ER and release the UPR depends on OR expression and nATF5 translation providing a potential explanation as to why the UPR is activated only at the initial stages of OR expression. In this vein, cellular changes induced by differentiation could also explain the sustained expression of Adcy3 long after nATF5 is cleared from the OSN nuclei. Although ATF5 is necessary for the priming and initiation of Adcy3 transcription, various transcription factors and signaling pathways that are active only in mOSNs could sustain the expression of this key regulator of OSN physiology.

Multiple layers of specificity in the OR-feedback signal

We recently showed that LSD1, the protein that allows the initiation of OR transcription, has to be downregulated upon OR expression for OR choice to be stabilized (Lyons et al., 2013). The realization that LSD1 downregulation requires Adcy3 expression, provided a connection between the OR signaling pathway and the OR feedback signal. However, this observation raised important questions regarding the signaling pathway that links OR to Adcy3 expression, because it was previously shown that a transgenic OR that does not couple to Gα proteins, and therefore cannot signal through Adcy3, is stably expressed in the MOE (Imai et al., 2006). The surprising discovery that ORs elicit their feedback and induce Adcy3 expression through the UPR, consolidates these findings and suggests that the OR protein *per se* can induce stable Adcy3 expression in a Gα-independent fashion. In other words, OR protein induces expression and not necessarily activation of Adcy3 during the induction of the feedback signal. Whether basal Adcy3 activity is sufficient- or other components of the OR signaling pathway are required for the maintenance of singular OR expression cannot be resolved from these experiments. However, the finding that ORs elicit their feedback through non-canonical GPCR signaling may provide an elegant solution to the issue of specificity in the detection of OR proteins by the OSN.

In addition to ORs, a number of non-olfactory GPCRs are also expressed in immature and mature OSNs. Moreover, large number or mammalian OR genes are pseudogenized and do not encode for intact OR protein (Niimura and Nei, 2005; Zhang and Firestein, 2002). Therefore the OSN faces the daunting task of recognizing and differentiating intact ORs from pseudogenous ones, as well as, from other members of the GPCR superfamily. Given the fact that the OR family is the largest and most diverse gene family, finding specificity in the context of variability is enormously complex and probably could not be reliably

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executed based on a single molecular test. Implementing multiple checkpoints for the recognition and endorsement of an OR protein by the OSN may be the solution for a vetting system that cannot rely on the preservation of a single, high affinity protein surface. The use of a two-step signaling process for feedback, relying on both UPR activation *and* relief, may provide the required specificity for the stable expression only of alleles encoding intact OR proteins.

The first step of OR detection by the OSN is the induction of the Perk signaling pathway. ORs may be highly enriched for select peptides acting as Perk ligands. The maintenance of these peptide ligands should aid in the initiation of feedback at the cost of immediate ER export, explaining why ORs, and chemoreceptors in general, tend to be retained in the ER or to be degraded upon heterologous expression, unlike most other GPCRs (Matsunami, 2005; Matsunami et al., 2009; Saito et al., 2004; Zhuang and Matsunami, 2007). The tendency of OR proteins to induce ER-stress may be also amplified by the extreme expression levels of OR genes in OSNs, providing an additional, quantitative trigger for UPR induction by OR proteins.

The second step of this pathway, the relief of ER stress, is as important as the induction of the UPR for the generation of a productive feedback signal, since Perk activation inhibits global OSN translation. We propose that relief of the UPR is a source of additional specificity for this system, because it relies upon proper transfer of the OR from the ER to the membrane. This step affords detail inspection of the receptor protein properties that could assure that the chosen OR is intact, properly folded and glycosylated, and that it has all the structural or sequence characteristics that define the OR family. OR-specific molecular chaperones, such as RTP1 and RTP2 (Saito et al., 2004), may distinguish intact ORs from OR pseudogenes and from non-OR GPCRs, providing an additional layer of specificity in the feedback signal. In agreement with this, pseudogene ORs, ORs with a mutation at the stereotypic N-terminal N-glycosylation site, or vomeronasal receptors replacing OR coding sequences cannot be stably expressed in olfactory neurons when transcribed from OR loci (Feinstein et al., 2004; Shykind et al., 2004). In contrast, the beta-2 adrenergic receptor, which has the stereotypic N-glycosylation site at its N-terminus and extended sequence homology with OR proteins can be stably expressed from an endogenous OR locus (Feinstein and Mombaerts, 2004). It is worth noting here that ATF5 is also highly expressed in the vomeronasal organ (VNO) suggesting that a similar signaling pathway may be employed by VRs and FPRs. Thus, a seductive model predicts that this limb of the UPR is universally triggered by highly transcribed chemoreceptors and relieved by the expression of chemoreceptor-specific chaperones that are different in each sensory organ, such as calreticulin chaperones in the VNO and RTP chaperones in the MOE (Dey and Matsunami, 2011; Saito et al., 2004).

In summary, our studies assign a novel biological function to the unfolded protein response, a conserved signaling pathway that evolved to maintain a productive ER folding environment. Unlike its typical role, however, in the MOE the UPR eventually stabilizes the transcription of the gene that induced this pathway, the chosen OR allele. Although co-opting the UPR to execute OR feedback required the recruitment of an ER-stress regulated transcription factor characterized by robust expression in the MOE, permutations of this concept may be generally applicable in cell type specification that relies upon GPCR or secretory protein expression. For example, a different arm of the UPR, acting through the kinase Ire-1 and the regulation of Xbp-1 splicing, is used in B-cells to promote ER expansion to accommodate the secretory cell fate transition (Iwakoshi et al., 2003a; Iwakoshi et al., 2003b; Reimold et al., 2001). Intriguingly, rapidly evolving gene families with stochastic, variegated, or mutually exclusive expression patterns encode, by and large, transmembrane or secreted proteins (Clowney et al., 2011). The diversification of these gene

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families, and the evolution of the myriad cell types defined by the expression of these genes, may have been enabled by the ability of the UPR to couple the activation or preservation of transcriptional programs to the appearance of a singularity.

Experimental Procedures

Mice and strains used

All mice were housed in standard conditions with a 12-hour light/dark cycle and access to food and water *ad libitum* and in accordance with the University of California IACUC guidelines. All strains were maintained on a mixed genetic background. Detailed information on the various mouse strains used is provided in Supplemental Experimental Procedures.

Immunofluorescence

IF was performed as previously described (Clowney et al. 2012). Antibodies used are listed in Table S1. Confocal images were collected with the Zeiss LSM 700. All image processing was carried out with ImageJ (NIH) or ImageJ in combination with R (Fig. 6B, Fig. S2A).

qRT-PCR and mRNA-seq

RNA for qRT-PCR or mRNA-seq libraries was prepared from whole MOE RNA as described previously (Magklara et al., 2011). qRT-PCR primers used are listed in Table S2. Sequencing libraries were prepared from whole MOE with standard methods using the ScriptSeq V2 kit (Epicentre). Detailed information can be found in Supplemental Experimental Procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References Cited

Barnea G, O'Donnell S, Mancia F, Sun X, Nemes A, Mendelsohn M, Axel R. Odorant receptors on axon termini in the brain. Science (New York, NY. 2004; 304:1468.

Buck L, Axel R. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. Cell. 1991; 65:175–187. [PubMed: 1840504]

Chess A, Simon I, Cedar H, Axel R. Allelic inactivation regulates olfactory receptor gene expression. Cell. 1994; 78:823–834. [PubMed: 8087849]

Clowney EJ, LeGros MA, Mosley CP, Clowney FG, Markenskoff-Papadimitriou EC, Myllys M, Barnea G, Larabell CA, Lomvardas S. Nuclear aggregation of olfactory receptor genes governs their monogenic expression. Cell. 2012; 151:724–737. [PubMed: 23141535]

Clowney EJ, Magklara A, Colquitt BM, Pathak N, Lane RP, Lomvardas S. High-throughput mapping of the promoters of the mouse olfactory receptor genes reveals a new type of mammalian promoter

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and provides insight into olfactory receptor gene regulation. Genome research. 2011; 21:1249–1259. [PubMed: 21705439]

Colquitt BM, Allen WE, Barnea G, Lomvardas S. Alteration of genic 5-hydroxymethylcytosine patterning in olfactory neurons correlates with changes in gene expression and cell identity. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110:14682–14687. [PubMed: 23969834]

Dey S, Matsunami H. Calreticulin chaperones regulate functional expression of vomeronasal type 2 pheromone receptors. Proceedings of the National Academy of Sciences of the United States of America. 2011; 108:16651–16656. [PubMed: 21933956]

Feinstein P, Bozza T, Rodriguez I, Vassalli A, Mombaerts P. Axon guidance of mouse olfactory sensory neurons by odorant receptors and the beta2 adrenergic receptor. Cell. 2004; 117:833–846. [PubMed: 15186782]

Feinstein P, Mombaerts P. A contextual model for axonal sorting into glomeruli in the mouse olfactory system. Cell. 2004; 117:817–831. [PubMed: 15186781]

Fleischmann A, Abdus-Saboor I, Sayed A, Shykind B. Functional interrogation of an odorant receptor locus reveals multiple axes of transcriptional regulation. PLoS biology. 2013; 11:e1001568. [PubMed: 23700388]

Hansen MB, Mitchelmore C, Kjaerulff KM, Rasmussen TE, Pedersen KM, Jensen NA. Mouse Atf5: molecular cloning of two novel mRNAs, genomic organization, and odorant sensory neuron localization. Genomics. 2002; 80:344–350. [PubMed: 12213205]

Harding HP, Novoa I, Zhang Y, Zeng H, Wek R, Schapira M, Ron D. Regulated translation initiation controls stress-induced gene expression in mammalian cells. Molecular cell. 2000a; 6:1099–1108. [PubMed: 11106749]

Harding HP, Zhang Y, Bertolotti A, Zeng H, Ron D. Perk is essential for translational regulation and cell survival during the unfolded protein response. Molecular cell. 2000b; 5:897–904. [PubMed: 10882126]

Imai T, Suzuki M, Sakano H. Odorant receptor-derived cAMP signals direct axonal targeting. Science (New York, NY. 2006; 314:657–661.

Iwakoshi NN, Lee AH, Glimcher LH. The X-box binding protein-1 transcription factor is required for plasma cell differentiation and the unfolded protein response. Immunol Rev. 2003a; 194:29–38. [PubMed: 12846805]

Iwakoshi NN, Lee AH, Vallabhajosyula P, Otipoby KL, Rajewsky K, Glimcher LH. Plasma cell differentiation and the unfolded protein response intersect at the transcription factor XBP-1. Nature immunology. 2003b; 4:321–329. [PubMed: 12612580]

Lewcock JW, Reed RR. A feedback mechanism regulates monoallelic odorant receptor expression. Proceedings of the National Academy of Sciences of the United States of America. 2004; 101:1069–1074. [PubMed: 14732684]

Lomvardas S, Barnea G, Pisapia DJ, Mendelsohn M, Kirkland J, Axel R. Interchromosomal interactions and olfactory receptor choice. Cell. 2006; 126:403–413. [PubMed: 16873069]

Lyons DB, Allen WE, Goh T, Tsai L, Barnea G, Lomvardas S. An epigenetic trap stabilizes singular olfactory receptor expression. Cell. 2013; 154:325–336. [PubMed: 23870122]

Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR, et al. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nature neuroscience. 2010; 13:133–140.

Magklara A, Lomvardas S. Stochastic gene expression in mammals: lessons from olfaction. Trends in cell biology. 2013; 23:449–456. [PubMed: 23689023]

Magklara A, Yen A, Colquitt BM, Clowney EJ, Allen W, Markenscoff-Papadimitriou E, Evans ZA, Kheradpour P, Mountoufaris G, Carey C, et al. An epigenetic signature for monoallelic olfactory receptor expression. Cell. 2011; 145:555–570. [PubMed: 21529909]

Matsunami H. Functional expression of Mammalian odorant receptors. Chemical senses. 2005; 30(Suppl 1):i95–i96. [PubMed: 15738214]

Matsunami H, Mainland JD, Dey S. Trafficking of mammalian chemosensory receptors by receptor-transporting proteins. Annals of the New York Academy of Sciences. 2009; 1170:153–156. [PubMed: 19686127]

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Nguyen MQ, Zhou Z, Marks CA, Ryba NJ, Belluscio L. Prominent roles for odorant receptor coding sequences in allelic exclusion. Cell. 2007; 131:1009–1017. [PubMed: 18045541]

Niimura Y, Nei M. Evolutionary dynamics of olfactory receptor genes in fishes and tetrapods. Proceedings of the National Academy of Sciences of the United States of America. 2005; 102:6039–6044. [PubMed: 15824306]

Pace U, Hanski E, Salomon Y, Lancet D. Odorant-sensitive adenylate cyclase may mediate olfactory reception. Nature. 1985; 316:255–258. [PubMed: 3927168]

Reimold AM, Iwakoshi NN, Manis J, Vallabhajosyula P, Szomolanyi-Tsuda E, Gravallese EM, Friend D, Grusby MJ, Alt F, Glimcher LH. Plasma cell differentiation requires the transcription factor XBP-1. Nature. 2001; 412:300–307. [PubMed: 11460154]

Ron D, Walter P. Signal integration in the endoplasmic reticulum unfolded protein response. Nature reviews. 2007; 8:519–529.

Saito H, Kubota M, Roberts RW, Chi Q, Matsunami H. RTP family members induce functional expression of mammalian odorant receptors. Cell. 2004; 119:679–691. [PubMed: 15550249]

Sammeta N, McClintock TS. Chemical stress induces the unfolded protein response in olfactory sensory neurons. The Journal of comparative neurology. 2010; 518:1825–1836. [PubMed: 20235094]

Sammeta N, Yu TT, Bose SC, McClintock TS. Mouse olfactory sensory neurons express 10,000 genes. The Journal of comparative neurology. 2007; 502:1138–1156. [PubMed: 17444493]

Scheuner D, Song B, McEwen E, Liu C, Laybutt R, Gillespie P, Saunders T, Bonner-Weir S, Kaufman RJ. Translational control is required for the unfolded protein response and in vivo glucose homeostasis. Molecular cell. 2001; 7:1165–1176. [PubMed: 11430820]

Serizawa S, Miyamichi K, Nakatani H, Suzuki M, Saito M, Yoshihara Y, Sakano H. Negative feedback regulation ensures the one receptor-one olfactory neuron rule in mouse. Science (New York, NY. 2003; 302:2088–2094.

Shykind BM, Rohani SC, O'Donnell S, Nemes A, Mendelsohn M, Sun Y, Axel R, Barnea G. Gene switching and the stability of odorant receptor gene choice. Cell. 2004; 117:801–815. [PubMed: 15186780]

Speake BK, Hemming FW, White DA. The effects of tunicamycin on protein glycosylation in mammalian and fungal systems. Biochem Soc Trans. 1980; 8:166–168. [PubMed: 7371958]

Wang SZ, Ou J, Zhu LJ, Green MR. Transcription factor ATF5 is required for terminal differentiation and survival of olfactory sensory neurons. Proceedings of the National Academy of Sciences of the United States of America. 2012; 109:18589–18594. [PubMed: 23090999]

Watatani Y, Ichikawa K, Nakanishi N, Fujimoto M, Takeda H, Kimura N, Hirose H, Takahashi S, Takahashi Y. Stress-induced translation of ATF5 mRNA is regulated by the 5'-untranslated region. The Journal of biological chemistry. 2008; 283:2543–2553. [PubMed: 18055463]

Zhang X, Firestein S. The olfactory receptor gene superfamily of the mouse. Nature neuroscience. 2002; 5:124–133.

Zhou D, Palam LR, Jiang L, Narasimhan J, Staschke KA, Wek RC. Phosphorylation of eIF2 directs ATF5 translational control in response to diverse stress conditions. The Journal of biological chemistry. 2008; 283:7064–7073. [PubMed: 18195013]

Zhuang H, Matsunami H. Synergism of accessory factors in functional expression of mammalian odorant receptors. The Journal of biological chemistry. 2007; 282:15284–15293. [PubMed: 17387175]

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Research Highlights

- Olfactory receptors cause ER stress and activate Perk in olfactory neurons
- Perk phosphorylates Eif2a, which induces translation of a nuclear isoform of ATF5
- ATF5 promotes Adenylyl cyclase 3 expression
- Adenylyl cyclase 3 relieves the ER stress and locks olfactory receptor choice

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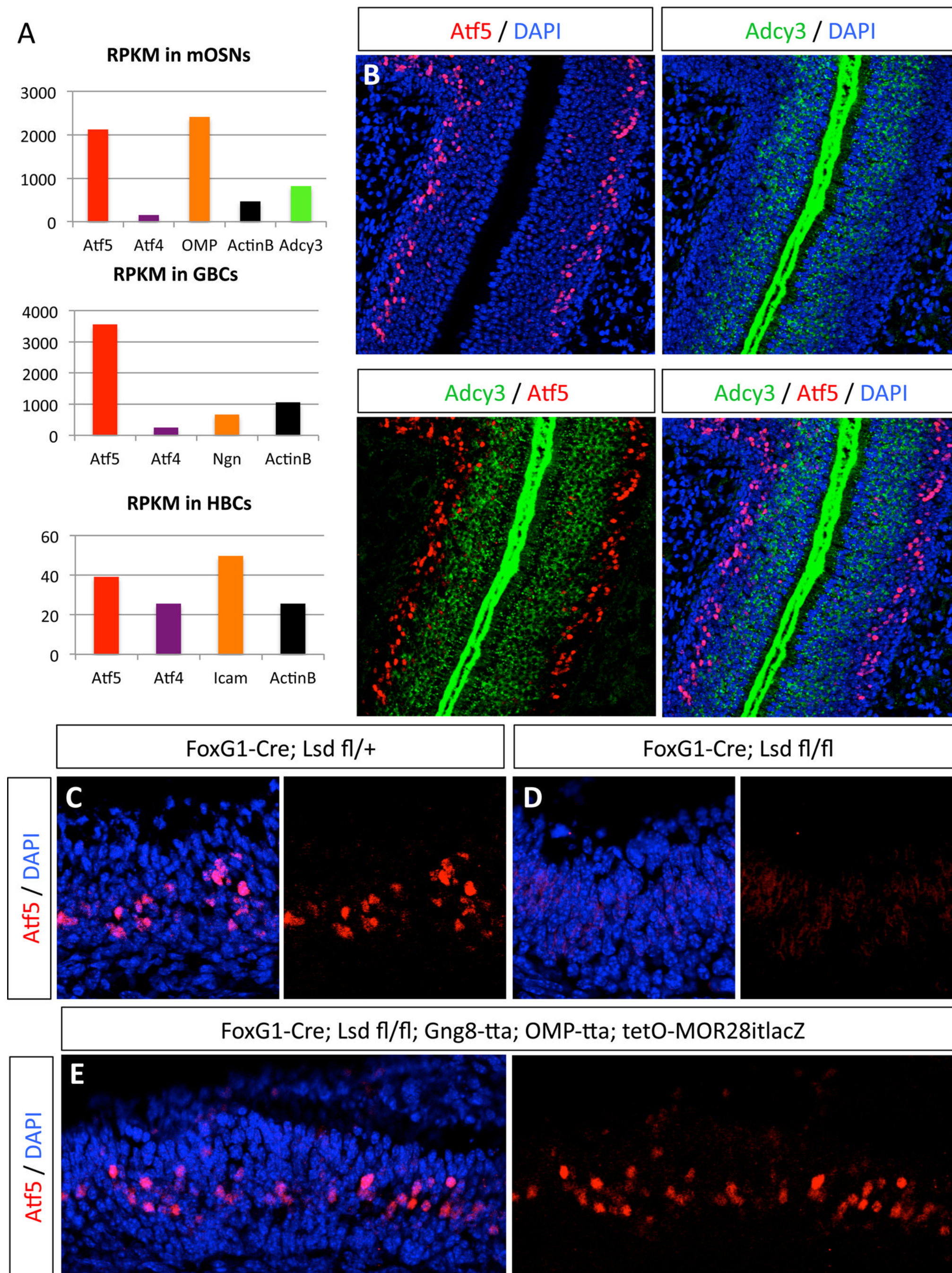

Figure 1.

(A) mRNA RPKM values for Atf5, Atf4, and developmental markers from cell populations isolated by fluorescence-activated cell sorting (FACS). HBCs: horizontal basal cells; GBCs: globose basal cells; mOSNs: mature olfactory sensory neurons.

(B) Atf5 immunofluorescence (IF, red), Adcy3 IF (green). DAPI nuclear stain (blue). Sections are from P40 animals. Specificity of Atf5 antibody is shown in Supplemental Figure S1A,B. ATF5 mRNA expression values shown in Supplemental Figure S1C.

(C) Atf5 IF in Foxg1-Cre; Lsd fl/+ at embryonic day 17 (E17) in

(D) Atf5 IF in Foxg1-Cre; Lsd fl/fl

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(E) Atf5 IF in Foxg1-Cre; Lsd fl/fl and transgenic OR rescue (Gng8tta; OMPtta; tetO-MOR28itlacZ) (Lyons et al., 2013).

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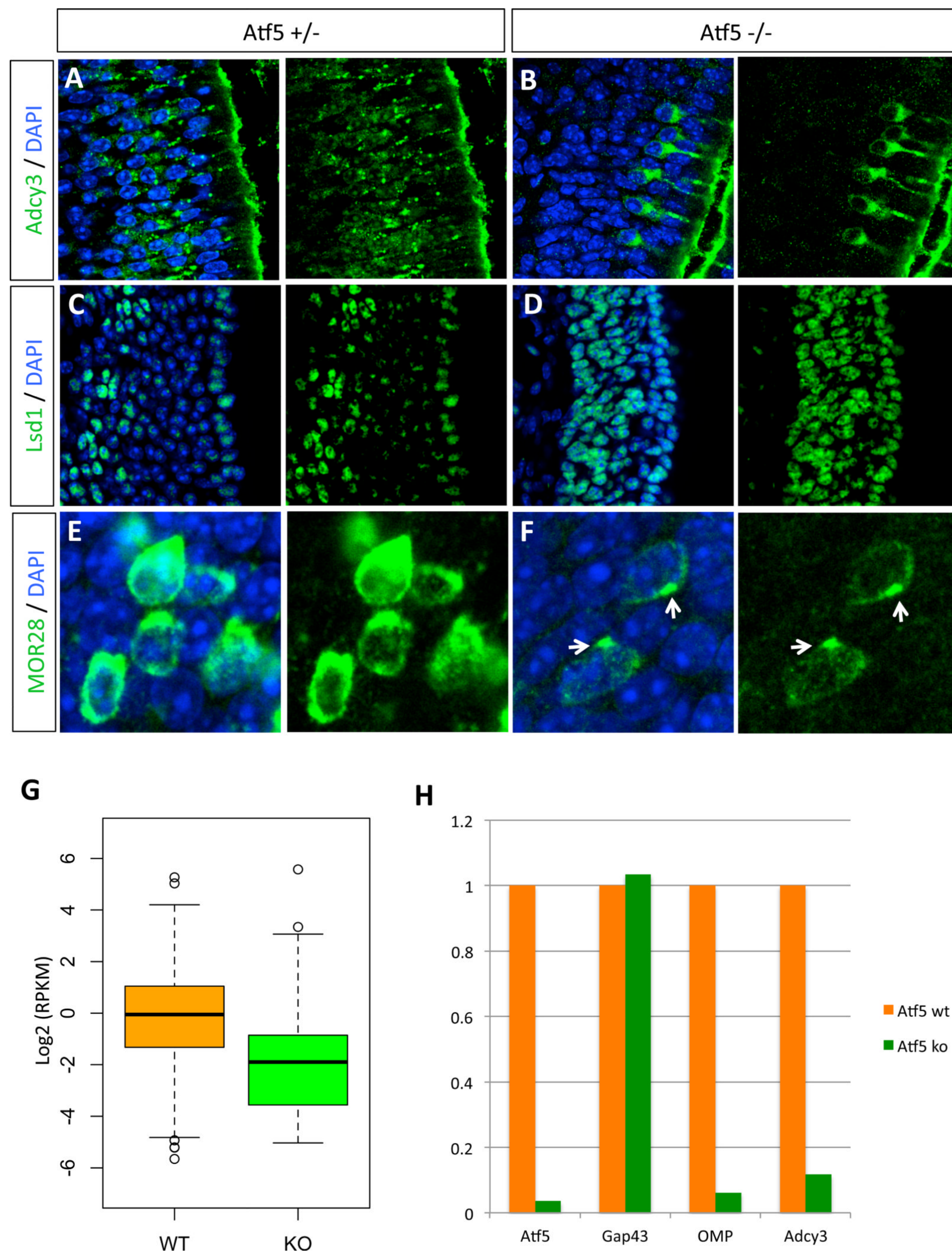

Figure 2.

(A–F) Sections from P40 Atf5+/– (left panels) and P40 –/– (right panels) stained with antibodies against Adcy3 (A–B), Lsd1 (C–D), or MOR28 (E–F). Shown with or without DAPI merge. Arrows in (F) point to ER regions with MOR28 aggregates only seen in ATF5 KO MOEs. Quantitation of the IF signal intensities for LSD1 and MOR28, as well as quantification of the number of MOR28+ OSNs are shown in Supplemental Figure S2A–C. (G) Boxplot summary of expression of refseq ORs from mRNA-seq on P40 Atf5 +/+ (orange, 1041 ORs detected) and Atf5 –/– (green, 939 ORs detected). Pseudogene ORs excluded.

(H) RPKM values normalized to wild-type for developmental markers. Atf5 +/+ shown in orange and Atf5 –/– in green. See Supplemental Figure S2D for expression levels of additional developmental markers.

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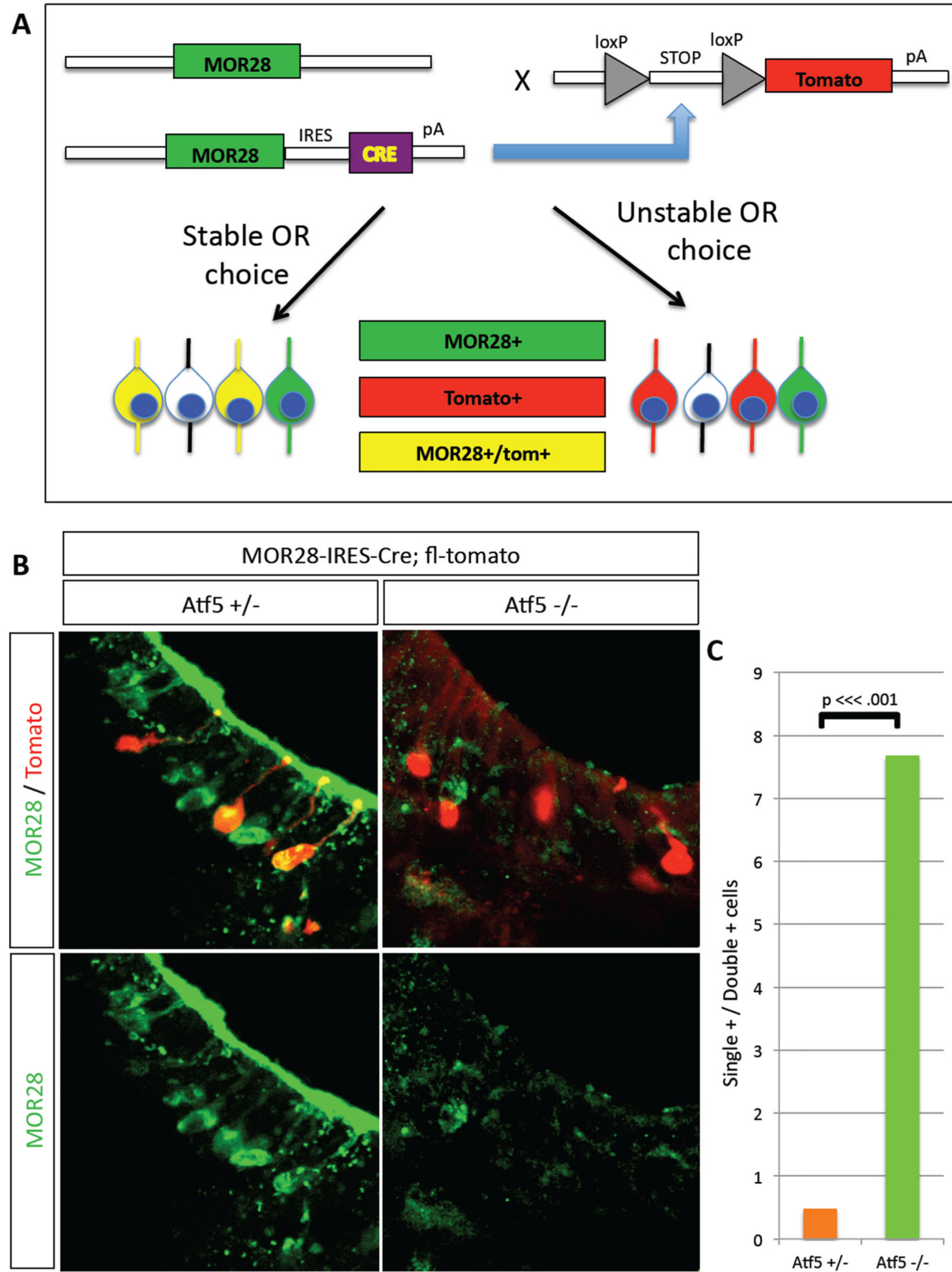

Figure 3.

(A) A genetic strategy to assay the stability of OR expression. One copy of the MOR28 gene also drives expression of Cre recombinase (MOR28-IRES-Cre), excising a stop signal from a Rosa lox-stop-lox-Tomato allele, permanently labeling the OSN with Tomato fluorescent protein. Cells with stable MOR28-IRES-Cre expression (left, yellow) are positive for Tomato and MOR28 as assayed by antibody staining, while cells with unstable MOR28-IRES-Cre expression (right, red), are positive for Tomato only. Cells that choose the wild type MOR28 allele are only green.

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(B) Sections from P40 MOR28-IRES-Cre; lox-stop-lox-Tomato; Atf5 +/– (left panels) or Atf5 –/– (right panels). MOR28 IF (green) alone (bottom) and with Tomato reporter (red) are shown (top).

(C) Quantification of gene switching from animals shown in (B). Data represented as percentage Tomato+/MOR28− cells over percentage Tomato+/MOR28+ cells. p-value <2.2e–16 (Fisher's test).

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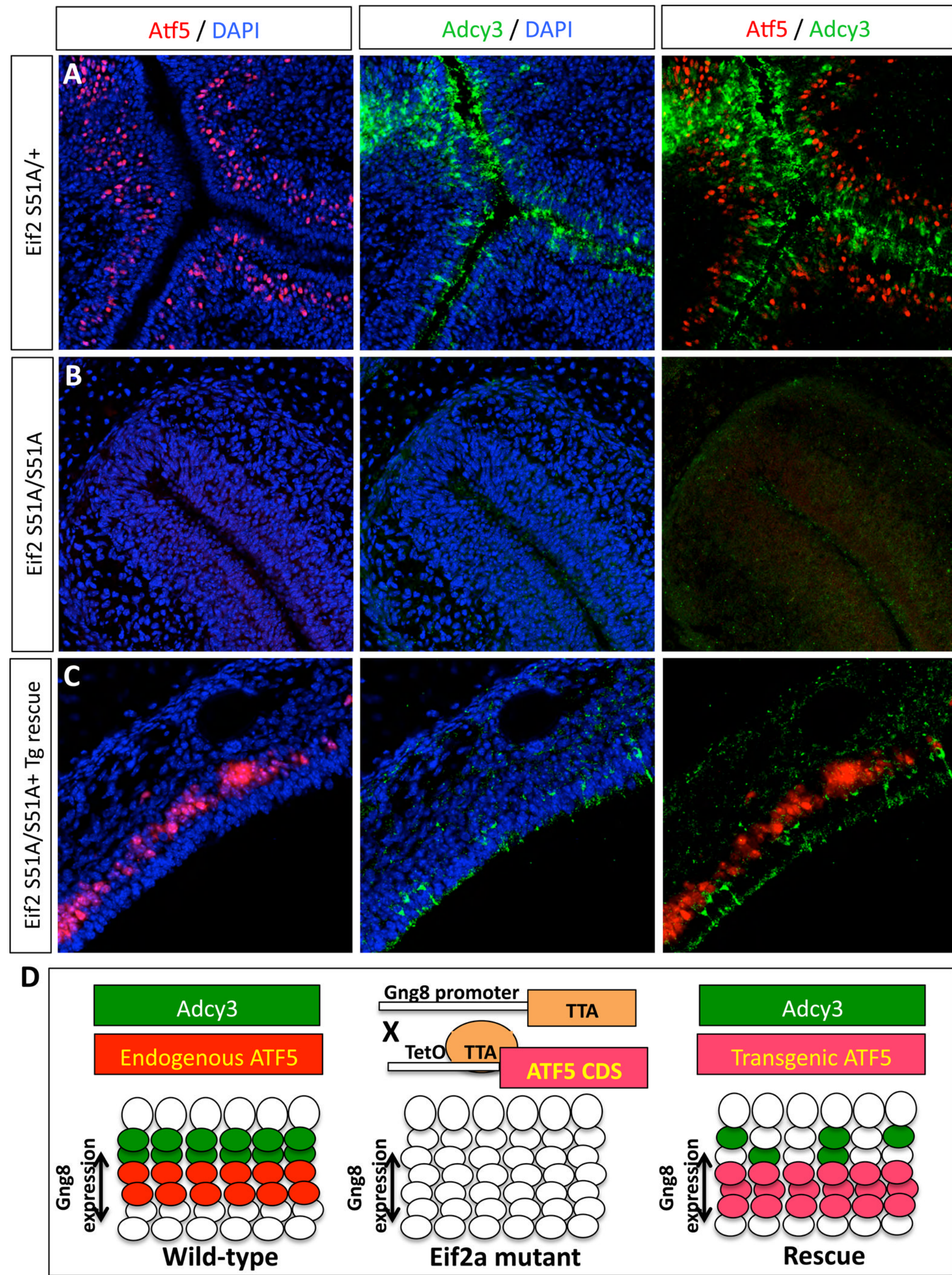

Figure 4.

(A) IF for Atf5 (red) with DAPI (left panel), IF for Adcy3 (green) with DAPI (middle panel) and IF for Atf5 and Adcy3 (right panel) in a section from a P0 Eif2 S51A/+ animal.

(B) IF for the same markers in a littermate Eif2 S51A/S51A animal. ATF5 mRNA levels in control and mutant MOEs shown in Supplemental Figure S3A.

(C) IF for the same markers in an Eif2 S51A/S51A animal with transgenic Atf5 rescue (Gng8-tta; tetO-Atf5).

(D) Genetic strategy for Atf5 transgenic rescue. Endogenous Atf5 protein is expressed just prior to Adcy3 expression (left). Eif2 phosphomutants (S51A/S51A) fail to express Atf5 or Adcy3 (middle). Transient expression of the Atf5 coding sequence under the control of

Gng8-tta results in a pattern of Atf5 expression slightly expanded towards the basal MOE, and partially rescues Adcy3 expression (see Supplemental Figure 3C for quantification).

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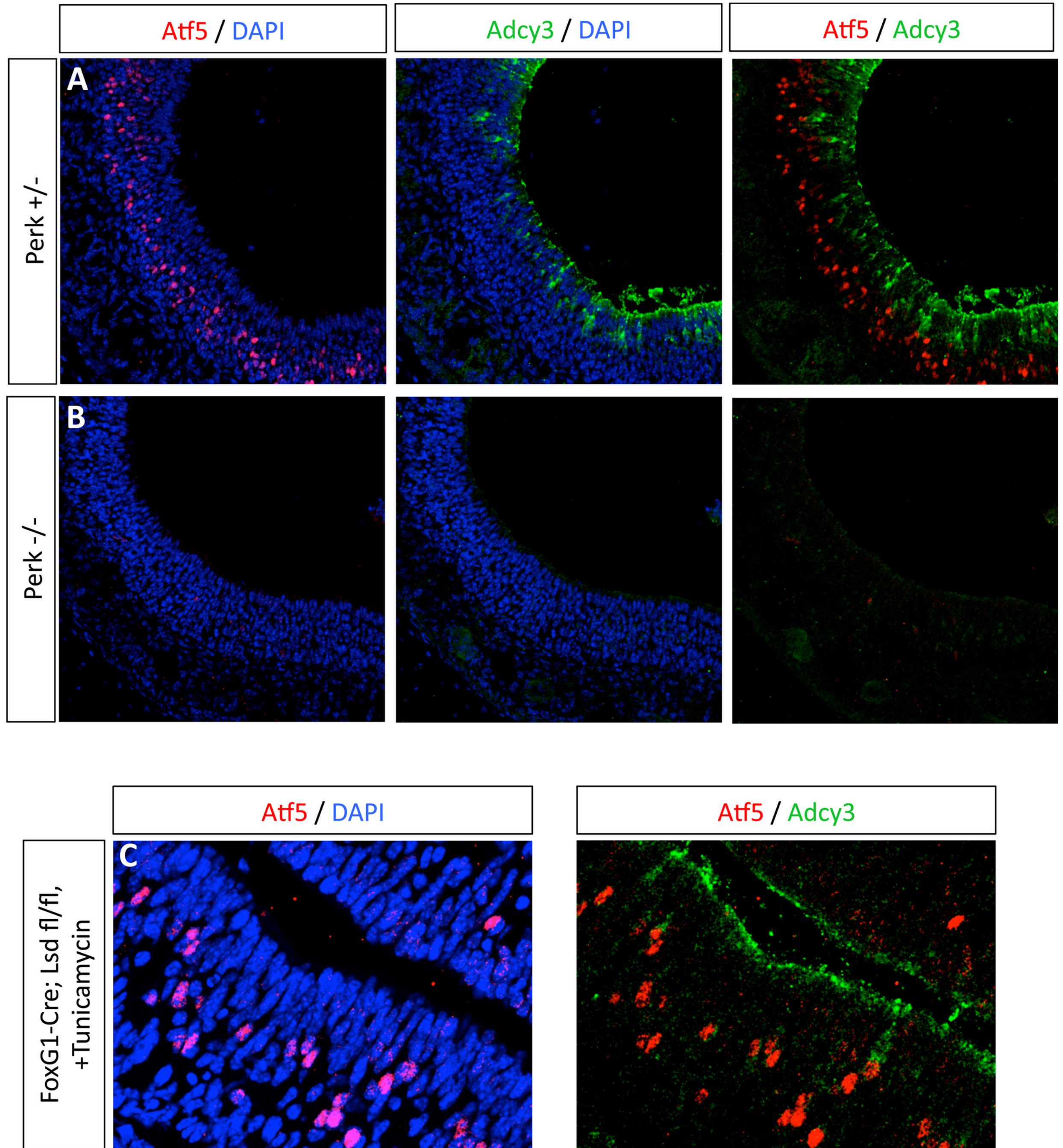

Figure 5.
(A) IF for Atf5 (red) with DAPI (left panel), IF for Adcy3 (green) with DAPI (middle panel) and IF for Atf5 and Adcy3 (right panel) in a section from a P0 Perk +/− animal.
(B) IF for the same markers in a Perk −/− littermate. Quantification of the numbers of ATF5-expressing cells and the intensity of IF signal shown in Supplemental Figure S4A,B.
(C) A female Lsd fl/fl mated to a male Lsd fl/+; Foxg1-Cre was given a single IP injection of tunicamycin at E16.5. At E17.5 pups were collected and sectioned. Shown is IF for Atf5 (red) and DAPI (left panel) and IF for Adcy3 (green) and Atf5 (red). For comparison with Foxg1-Cre; Lsd fl/fl or Lsd fl/+, see Figure 1 and for quantification of the numbers of ATF5-expressing cells see Supplemental Figure S4A.

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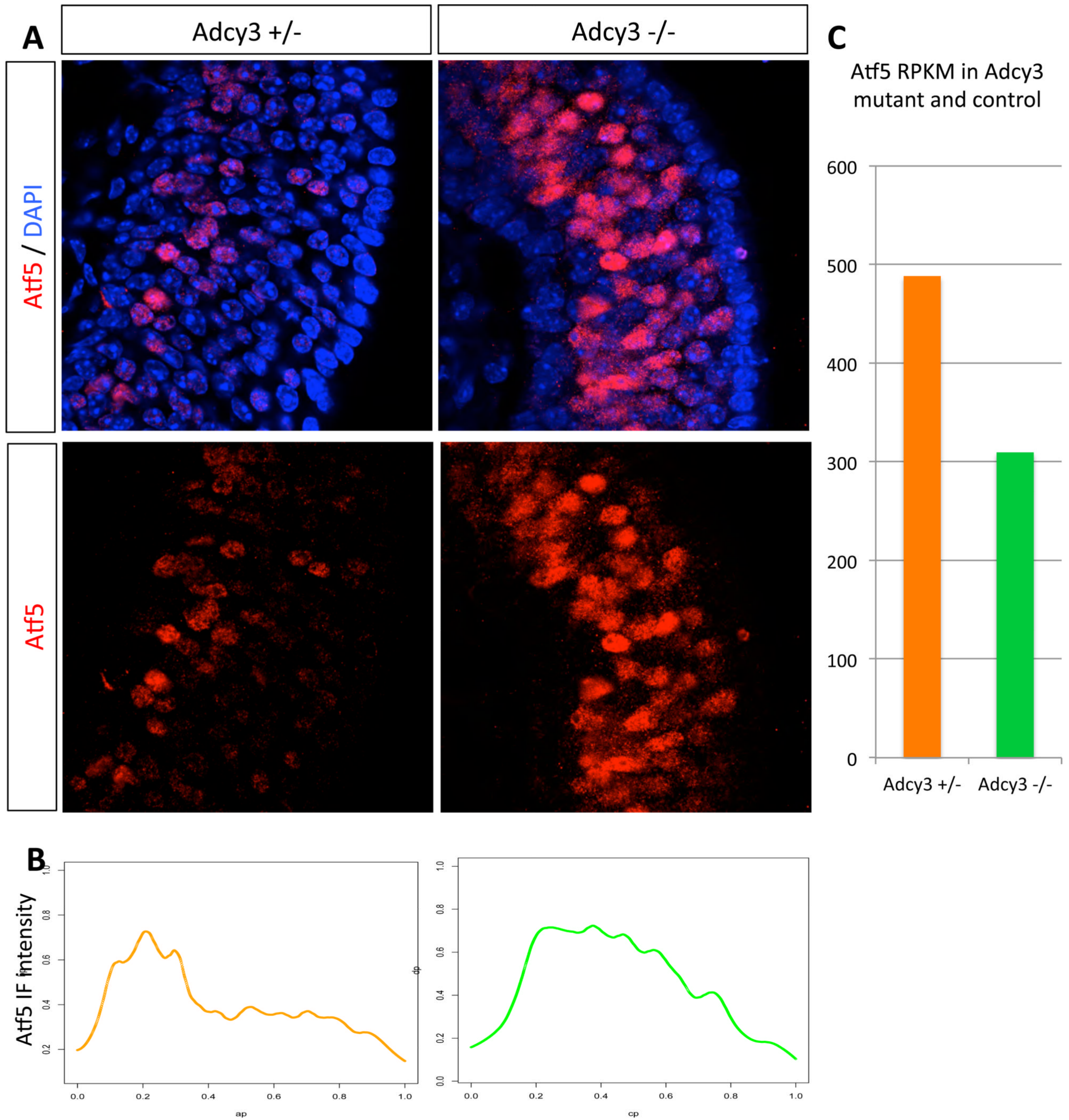

Figure 6.

(A) IF for Atf5 (red) with and without DAPI merge in Adcy3 +/– (left panels) and Adcy3 –/– (right panels).

(B) Quantification of Atf5 fluorescence intensity in a section from Adcy3 +/– or Adcy3 –/– animals. Shown as % basal to apical position vs. % maximum intensity (see methods). Raw data shown as scatterplot in background and locally weighted scatterplot smoothing shown in orange (Adcy3+/–) or green (Adcy3 –/–)

(C) RPKM values for Atf5 mRNA in Adcy3 +/– and Adcy3 –/–

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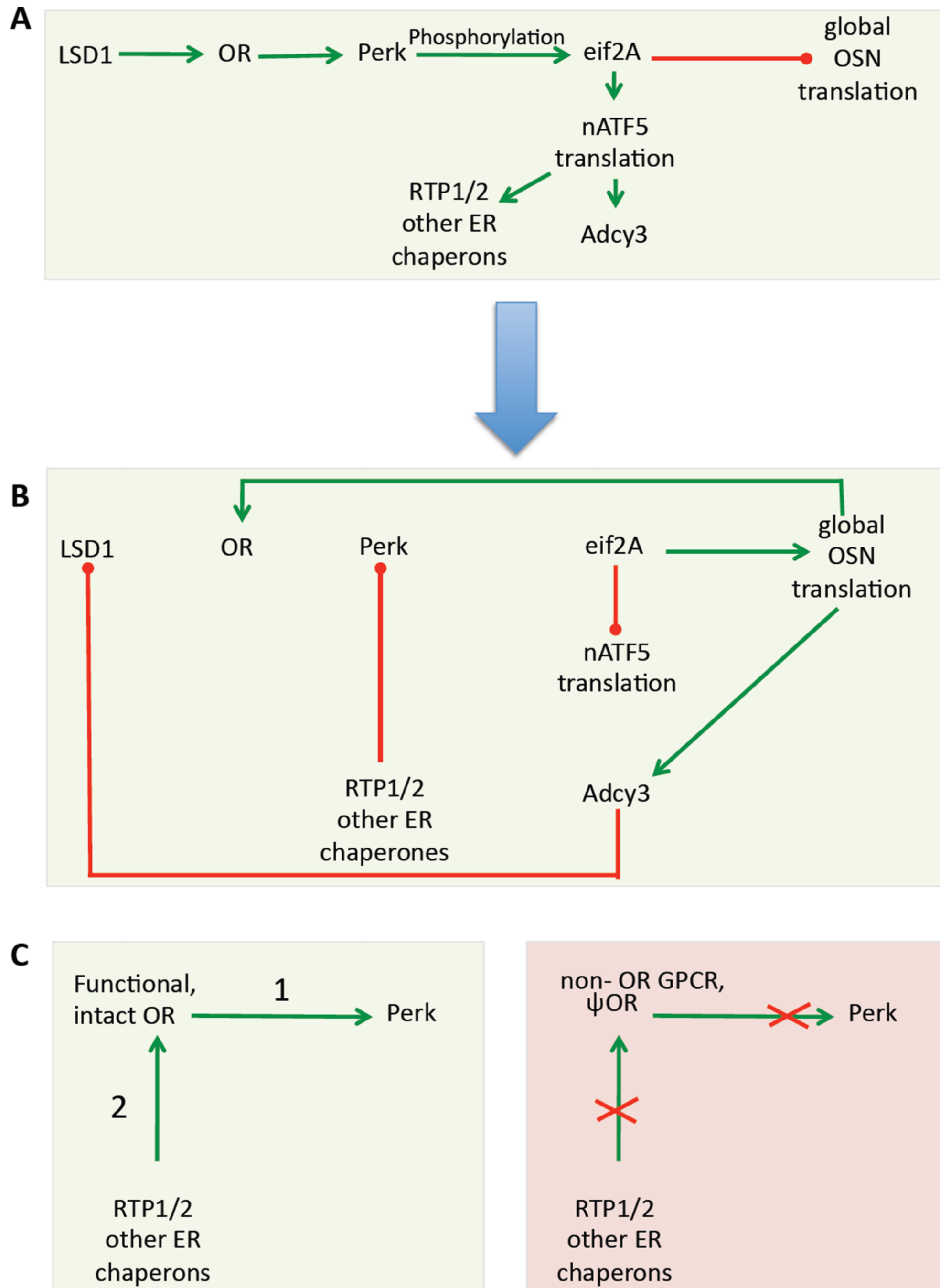

Figure 7.

(A) A model for the generation of the OR feedback signal: Lsd1 transcriptionally activates an OR, which is co-translationally detected by Perk in the endoplasmic reticulum. OR-Perk interaction activates Perk, which then phosphorylates eif2α, resulting in a global pause in translation initiation and a selective increase in nuclear Atf5 translation. Atf5 activity initiates Adcy3 transcription, and according to our RNAseq analysis also activates transcription of OR chaperones RTP1 and RTP2 (Supplemental Figures S2D).

(B) nATF5-dependent upregulation of Adcy3 and OR-specific chaperones relieves the ER stress and restores global translation in the OSN. Although this leads to an increase of OR and Adcy3 protein levels, it stops the translation of nATF5 isoform clearing this protein

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from the nucleus. Increased OR and Adcy3 levels also cause downregulation of LSD1 (Lyons et al., 2013) preventing OR switching and stabilizing OR choice.
(C) A two-step model of feedback explains its specificity for ORs by providing two independent tests: 1) induction of UPR, and 2) relief of the UPR. Intact ORs (left) that pass both tests are stably expressed for the life of the neuron; non olfactory GPCRs and/or pseudogene ORs may fail to activate Perk allowing the process of OR choice to continue until an intact OR is expressed. At a second level of specificity a GPCR or pseudogene OR that passes the first test may not be recognized by OR-specific chaperones causing prolonged ER stress and sustained LSD1 expression, eventually allowing activation of an OR allele and/or OR gene switching.