A carboxy-terminal domain determines the subunit specificity of KCNQ K⁺ channel assembly

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Mutations in KCNQ K⁺ channel genes underlie several human pathologies. KCNQ α -subunits form either homotetramers or hetero-oligomers with a restricted subset of other KCNQ α -subunits or with KCNE β -subunits. KCNQ1 assembles with KCNE β -subunits but not with other KCNQ α -subunits. By contrast, KCNQ3 interacts with KCNQ2, KCNQ4 and KCNQ5. Using a chimaeric strategy, we show that a cytoplasmic carboxy-terminal subunit interaction domain (*sid*) suffices to transfer assembly properties between KCNQ3 and KCNQ1. A chimaera (KCNQ1-*sid*_{Q3}) carrying the *si* domain of KCNQ3 within the KCNQ1 backbone interacted with KCNQ2, KCNQ3 and KCNQ4 but not with KCNQ1. This interaction was shown by enhancement of KCNQ2 currents, testing for dominant-negative effects of pore mutants, determining its effects on surface expression and co-immunoprecipitation experiments. Conversely, a KCNQ3-*sid*_{Q1} chimaera no longer affects KCNQ2 but interacts with KCNQ1. We conclude that the *si* domain suffices to determine the subunit specificity of KCNQ channel assembly.

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INTRODUCTION

Mutations in four of the five human *KCNQ* genes lead to inherited diseases. Dominant-negative *KCNQ1* mutations underlie some forms of the long-QT syndrome (Wang *et al.*, 1996) which is associated with cardiac arrhythmias. Patients carrying recessive *KCNQ1* mutations on both alleles suffer additionally from congenital deafness in the Jervell and Lange-Nielsen syndrome (Neyroud *et al.*, 1997). Mutations in either *KCNQ2* or *KCNQ3* lead to dominantly inherited benign familial neonatal convulsions (BFNC), a neonatal epilepsy (Biervert *et al.*, 1998; Charlier *et al.*, 1998; Singh *et al.*, 1998). Mutations in *KCNQ4* cause DFNA2, a form of dominant progressive hearing loss (Kubisch *et al.*, 1999).

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Although they can form functional homotetramers, under physiological circumstances KCNQ proteins most often assemble into heteromeric channels containing different KCNQ α -subunits, or associate with KCNE β -subunits. Thus, KCNQ1 assembles with the single transmembrane-spanning β -subunit KCNE1 (Barhanin *et al.*, 1996; Sanguinetti *et al.*, 1996) to form the slowly activating I_{Ks} current in the heart, and interacts with KCNE3 to mediate the basolateral K⁺-conductance in colonic crypt cells (Schroeder *et al.*, 2000b). Whereas KCNQ1 might be unable to form heteromers with other KCNQ proteins, KCNQ3 forms functional heteromers with KCNQ2 (Schroeder *et al.*, 1998; Wang *et al.*, 1998), KCNQ4 (Kubisch *et al.*, 1999) and KCNQ5 (Schroeder *et al.*, 2000a). Both KCNQ2/KCNQ3 heteromers and channels containing KCNQ5 probably underlie the neuronal M-current (Wang *et al.*, 1998; Schroeder *et al.*, 2000a), an important regulator of neuronal excitability.

We set out to identify structures determining the subunit specificity of KCNQ subunit assembly. As previous experiments hinted at a role of the cytoplasmic carboxy terminus in homomeric channel assembly (Schmitt *et al.*, 2000; Schwake *et al.*, 2000), we constructed and analysed chimaeric channels in which C-terminal domains were swapped between KCNQ1 and KCNQ3.

RESULTS

KCNQ1 seems unable to associate with other KCNQ α -subunits, whereas KCNQ3 can form functional hetero-oligomers with KCNQ2, KCNQ4 and KCNQ5 (Jentsch, 2000). We tried to confer broad hetero-oligomerization properties on KCNQ1 by replacing C-terminal segments of KCNQ1 by the corresponding stretches of KCNQ3 (Fig. 1). By taking the segment between KCNQ3 residues 535 and 650, we included both the highly conserved A-domain (Jentsch, 2000; Schwake *et al.*, 2000) and a further C-terminal segment corresponding to a domain important for KCNQ1 assembly (Schmitt *et al.*, 2000). We named this construct KCNQ1-*sid*_{Q3}, where *sid* denotes subunit interaction domain. This construct was tested in *Xenopus* oocytes for interactions with either KCNE1 or various KCNQ α -subunits. Figure 2 shows typical currents of *Xenopus* oocytes previously injected with KCNQ1 (Fig. 2A) and chimaeric KCNQ1-*sid*_{Q3} RNA (Fig. 2B). The KCNQ1-*sid*_{Q3} construct yielded K⁺ currents that resembled wild-type KCNQ1 currents but had reduced amplitudes and showed pronounced inactivation at depolarizing potentials.

Fig. 1 | Alignment of C-terminal sequences of KCNQ1 and KCNQ3 according to Charlier et al. (1998). The *si* domains from KCNQ3 and KCNQ1 are indicated by grey and white boxes, respectively. The A-part and H-part of the *si* domain for the construction of partial chimaeras are also shown. Conserved amino acids are shown in bold.

Because KCNQ1 associates with the β -subunit KCNE1 in the heart to yield the slowly activating I_{Ks} current (Barhanin et al., 1996; Sanguinetti et al., 1996), we compared the effect of KCNE1 on KCNQ1 (Fig. 2C) with that on KCNQ1-*sid*_{Q3} (Fig. 2D). KCNE1 increased KCNQ1 currents and slowed activation. KCNQ1-*sid*_{Q3}/KCNE1 activated faster than wild-type KCNQ1/KCNE1, and currents saturated after a few seconds. The current-voltage relation was shifted by at least +30 mV compared with wild-type heteromers. KCNQ1-*sid*_{Q3} currents were not increased by KCNE1 up to +60 mV (compare the dashed current trace in Fig. 2C with the upper trace in Fig. 2B). This indicates that KCNQ1-*sid*_{Q3} was still able to interact with KCNE1. However, the resulting gating properties were different, indicating that the exchanged *si* domain also contains sequences important for channel gating and/or KCNE1 interactions, as also suggested by the studies of Chouabe et al. (2000).

We then tested whether KCNQ1-*sid*_{Q3} could replace KCNQ3 in stimulating currents of KCNQ2 by heteromer formation (Schroeder et al., 1998; Wang et al., 1998; Yang et al., 1998). Figure 3A and D shows currents of KCNQ2 and KCNQ3, respectively. When the KCNQ1-*sid*_{Q3} construct was co-expressed with KCNQ2, currents increased by a factor of ~8 (Fig. 3B and G), which is close to the factor of ~10 found for KCNQ2/KCNQ3 co-expression (Schroeder et al., 1998). Currents obtained by co-expressing KCNQ1-*sid*_{Q3} with KCNQ2 resembled those from wild-type KCNQ2/3 heteromers, although with a slower activation rate (compare Fig. 3B and C). Co-expressing KCNQ1-*sid*_{Q3} with KCNQ3 also moderately increased currents (Fig. 3E; see also Fig. 4A). Tail current analysis showed a significant shift ($P < 0.005$, paired *t*-test) of the activation curve for KCNQ1-*sid*_{Q3}/KCNE1 (Fig. 3F) and KCNQ1-*sid*_{Q3}/KCNE1 (Fig. 3H) to depolarizing potentials compared with KCNQ2 or KCNQ3 homomers and KCNQ2/KCNQ3 heteromers (see Table 1). These data indicate that the KCNQ1-*sid*_{Q3} construct interacts functionally with KCNQ2 and KCNQ3.

Next we constructed chimaeras in which only parts (A-part and H-part; Fig. 1) of the *si* domain were exchanged. When only the highly homologous A-part of the *si* domain was replaced, KCNQ2 currents were not enhanced (data not shown), whereas replacing the H-part of the *si* domain led to a significant, but comparatively small, threefold increase upon co-expression with KCNQ2 (Fig. 3G). We did not try to narrow down the borders of the *si* domain, and used the KCNQ1-*sid*_{Q3} construct for our further studies.

To probe α -subunit interactions further, we constructed a KCNQ1-*sid*_{Q3} mutant that contained the dominant-negative pore mutation G314S (Wollnik et al., 1997). We co-expressed this mutant (termed KCNQ1-*sid*_{Q3}(GS)) with other KCNQs. Co-expression of KCNQ1-*sid*_{Q3}(GS) with KCNQ2 decreased KCNQ2 currents by 70%, showing a dominant-negative effect (Fig. 3G). When KCNQ3 was co-expressed with KCNQ1-*sid*_{Q3}, currents were increased about threefold, whereas KCNQ1-*sid*_{Q3}(GS) exerted a strong dominant-negative effect and suppressed currents to background levels (Fig. 4A). KCNQ1-*sid*_{Q3}(GS) also had a small dominant-negative effect on KCNQ4: currents were decreased by ~70% (Fig. 4B).

The experiments shown in Fig. 4C demonstrate that the G314S pore mutant exerts a dominant-negative effect in a 1:1 co-injection scheme with KCNQ1 (compare the first two columns). When the *si* domain of the dominant-negative construct was exchanged with that of KCNQ3 in the KCNQ1-*sid*_{Q3}(GS) construct, the dominant-negative effect was lost because the 50% current level (Fig. 4C, last column) was due to the fact that only half the amount of wild-type KCNQ1 RNA had been injected. Thus, replacing the *si* domain of KCNQ1 by that of KCNQ3 destroyed its ability to associate with KCNQ1.

We also constructed an 'inverse' chimaera (termed KCNQ3-*sid*_{Q1}) to test whether the *si* domain of KCNQ1 also functions in the context of KCNQ3. Although KCNQ3 currents were already very small, those mediated by KCNQ3-*sid*_{Q1} were nearly indistinguishable from background currents (data not shown). Whereas KCNQ3 led to a large increase in currents when co-expressed with KCNQ2, this was not observed with KCNQ3-*sid*_{Q1}. Furthermore, when the dominant-negative pore mutation G318S was inserted into KCNQ3-*sid*_{Q1} (in the KCNQ3-*sid*_{Q1}(GS) construct), no dominant-negative effect on KCNQ2 was observed (Fig. 5A). Both types of experiment suggest that the exchange of the *si* domain destroyed the ability of KCNQ3 to interact with KCNQ2.

Fig. 2 | Current recordings from oocytes injected with cRNAs of KCNQ1 (A), KCNQ1-*sid*_{Q3} (B), KCNQ1 + KCNE1 (C) and KCNQ1-*sid*_{Q3} + KCNE1 (D). Dashed lines in C and D indicate currents after a pulse to +60 mV. Insets show the applied voltage protocols.

Fig. 3 | Current characteristics of various KCNQ channel constructs/co-injections. **A–E**, Representative current recordings from oocytes expressing KCNQ2 (**A**), KCNQ2 + KCNQ1-sid_{Q3} (**B**), KCNQ2 + KCNQ3 (**C**), KCNQ3 (**D**) and KCNQ3 + KCNQ1-sid_{Q3} (**E**). **F, H**, The voltage dependence of normalized tail current amplitudes (each data set from five oocytes). The resulting $V_{0.5}$ values are summarized in Table 1. **G**, Effect of co-expressing the KCNQ1-sid_{Q3} chimera, the KCNQ1-sid_{Q3}(G314S) pore mutant and the partial chimera KCNQ1-H_{Q3} on KCNQ2. Average current amplitudes at the end of a 2.5-s test pulse to +40 mV from oocytes (co-)injected with cRNAs as indicated are shown ($n > 10$ oocytes for each column).

In contrast, KCNQ3-sid_{Q1}(GS) exerted a dominant-negative effect on currents of KCNQ1/KCNQ1 heteromers (the I_{Ks} current) (Fig. 5B) without changing its slow activation by depolarization (data not shown).

We wished to confirm the role of the *si* domain in channel assembly at the biochemical level and performed co-immunoprecipitation experiments using KCNQ1-sid_{Q3} and KCNQ1 or KCNQ2. Figure 6 shows that Flag (DYKDDDDK)-tagged KCNQ2 co-precipitated with HA (haemagglutinin)-tagged KCNQ1-sid_{Q3} as efficiently as with HA-tagged KCNQ3. By contrast, there was only a very faint signal when co-immunoprecipitation was attempted with HA-tagged

KCNQ1. Thus, the *si* domain is a major determinant for KCNQ subunit assembly.

The strong current increase upon KCNQ2/KCNQ3 co-expression is due primarily to an increased surface expression (Schwake et al., 2000). We therefore investigated whether co-expression of KCNQ1-sid_{Q3} affected the surface expression of various KCNQ subunits.

The KCNQ1-sid_{Q3} chimera was nearly as efficient as KCNQ3 in stimulating the surface expression of epitope-tagged KCNQ2 (Fig. 7A). Its 80% efficiency (compared with KCNQ3) agrees well with the eightfold current increase found for KCNQ2/KCNQ1-sid_{Q3} co-injection when compared with the tenfold increase for

Table 1 | $V_{0.5}$ values for different KCNQ homomers and heteromers (five oocytes each)

Homomer or heteromer	$V_{0.5}$ (mV)
KCNQ1- <i>sid</i> _{Q3}	-23.9 ± 2.4
KCNQ3	-50.2 ± 2.2
KCNQ3 + KCNQ1- <i>sid</i> _{Q3}	-32.6 ± 1.3
KCNQ2	-34.6 ± 5.8
KCNQ2 + KCNQ1- <i>sid</i> _{Q3}	-19.2 ± 3.7
KCNQ2 + KCNQ3	-37.4 ± 1.5

KCNQ2/KCNQ3 (Schroeder et al., 1998). Co-expression with KCNQ1 did not affect the surface expression of HA-tagged-KCNQ2 (KCNQ2(HA)). In the inverse experiment, we tested whether the surface expression of KCNQ1-*sid*_{Q3} could be stimulated by KCNQ2. We tagged KCNQ1 and KCNQ1-*sid*_{Q3} constructs with an HA epitope in the extracellular loop between transmembrane spans S1 and S2. The surface expression of HA-tagged KCNQ1-*sid*_{Q3} construct was lower than that of HA-tagged KCNQ1. Upon co-expression of KCNQ1-*sid*_{Q3}(HA) with KCNQ2, surface expression increased about tenfold (Fig. 7B), an increase that was almost identical to the increase in surface expression of the HA-tagged KCNQ3 upon KCNQ2 co-expression (Schwake et al., 2000). Co-expressing KCNQ1-*sid*_{Q3}(HA) with KCNQ3 decreased surface expression by 50%, because only half of the channel subunits present in the plasma membrane carry an epitope under these conditions. We should note that tagging with an HA epitope did not alter biophysical channel properties, although we generally observed 20–40% lower current levels (data not shown).

DISCUSSION

Two types of domain involved in K⁺-channel assembly have been identified. Members of the K_v channel group contain an amino-terminal tetramerization domain (termed T1) that is important for functional expression (Li et al., 1992), and in some K_v1 channels serves primarily to prevent assembly with incompatible subunits (Papazian, 1999). In contrast, C-terminal sequences have been suggested to be required for assembly of functional rat *ether a go-go* (Ludwig et al., 1997), KCNQ1 (Schmitt et al., 2000) and inward rectifier (Tinker et al., 1996) K⁺ channels.

Previous work has already implicated the C-terminus in KCNQ channel assembly. A BFNC missense mutation in KCNQ2 (Biervert et al., 1998), truncating large parts of the KCNQ2 C terminus (before the A-part of the *si* domain from Fig. 1), resulted in a lack of plasma membrane expression and failure to interact with KCNQ3 (Schwake et al., 2000). The region between amino acids 590 and 620 was implicated in KCNQ1 assembly (Schmitt et al., 2000). This region encompasses a leucine zipper motif to which the scaffold protein yotiao binds in KCNQ1 (between amino acids 588 and 616), rendering KCNQ1 susceptible to β -adrenergic receptor-mediated regulation (Marx et al., 2002). This motif is absent from other KCNQ channels. Hence, in addition to its significance for specific subunit assembly, this region might be important for other protein–protein interactions of some KCNQ proteins.

We have investigated the distinct heteromerization properties of KCNQ K⁺ channels by replacing amino-acid residues 530–620 of KCNQ1 by the homologous region of KCNQ3 (residues 535–650). The exchanged region encompasses a highly conserved stretch (A-part; see Fig. 1) followed by a region of rather poor homology. Analysis of the KCNQ1-*sid*_{Q3} chimaera demonstrates that transplanting the *si* domain from KCNQ3 to KCNQ1 suffices to transfer homomeric and heteromeric assembly properties from KCNQ3 to KCNQ1. The H-part of the *si* domain, previously recognized to be important for KCNQ1 assembly (Schmitt et al., 2000), confers only basal, inefficient assembly properties; it also requires the highly conserved A-domain for full activity.

Evidence is drawn from several observations. First, KCNQ1-*sid*_{Q3} enhanced currents upon co-expression with KCNQ2 about eightfold, which is close to the factor of ~10 reported for KCNQ2/KCNQ3 co-expression (Schroeder et al., 1998; Wang et al., 1998; Yang et al., 1998). For both KCNQ2 and KCNQ3, channel properties resulting from co-expression with KCNQ1-*sid*_{Q3} differed from properties of the respective homomers. Second, co-immunoprecipitation experiments revealed that KCNQ1-*sid*_{Q3} assembles with KCNQ2 but not with KCNQ1. Third, co-expression of the dominant-negative KCNQ1-*sid*_{Q3}(GS) construct suppressed KCNQ2, KCNQ3 and KCNQ4 currents, whereas no dominant-negative effect on KCNQ1 was observed. Fourth, an ‘inverse’ chimaera (KCNQ3-*sid*_{Q1}), in which the *si* domain of KCNQ1 was inserted into KCNQ3, failed to stimulate currents when co-expressed with KCNQ2. A KCNQ3-*sid*_{Q1} construct carrying the KCNQ3 G318S pore mutation did not suppress KCNQ2

Fig. 4 | Effect of KCNQ1-*sid*_{Q3} and the KCNQ1-*sid*_{Q3}(G314S) pore mutant on KCNQ3 (A), KCNQ4 (B) and KCNQ1 (C). Average current levels at the end of a 2.5-s test pulse to +40 mV from oocytes (co-)injected with KCNQ cRNAs as indicated are shown. Values are means $\pm$ s.e. ($n \geq 10$ oocytes from two frogs).

Fig. 5 | Effect of the ‘inverse’ chimera KCNQ3-*sid*_{Q1} and the KCNQ3-*sid*_{Q1}(G318S) pore mutant on KCNQ2 (A) and KCNQ1 + KCNE1 (*I*_h) (B). Currents at the end of a 2.5-s test pulse to +40 mV from oocytes (co-)injected with cRNAs as indicated are shown. Values are means ± s.e., which in (A) are normalized to the level of KCNQ2 currents ($n \geq 10$ oocytes from two or three frogs).

currents, but instead decreased KCNQ1/KCNE1 currents. This demonstrates the general importance of *si*-domain-mediated interaction in KCNQ channel assembly. Finally, evidence is drawn from the strong enhancement of surface expression when KCNQ1-*sid*_{Q3} was co-expressed with KCNQ2. Surface expression of HA-tagged KCNQ2 was stimulated about threefold upon co-expression with KCNQ1-*sid*_{Q3}, which is somewhat less than the factor of 4 observed for KCNQ2(HA)/KCNQ3 co-expression (Schwake et al., 2000), in line with the smaller current augmentation. Because the subunit stoichiometry, unitary conductances and open probabilities of heteromeric channels are unknown, a direct correspondence of currents and surface expression levels cannot be expected. Assuming an average 2:2 stoichiometry, only two subunits would carry an epitope in a heteromer, giving an additional factor of 2 in surface expression. When an HA-tagged KCNQ1-*sid*_{Q3} was co-expressed with KCNQ2, surface expression was increased by the same amount (about tenfold)

Fig. 6 | Co-assembly of the HA-tagged KCNQ1-*sid*_{Q3} construct with Flag-tagged KCNQ2. Protein complexes obtained from oocytes expressing various KCNQ subunits were co-immunoprecipitated with anti-HA antibody, separated by SDS-PAGE and detected by western blot analysis with an anti-Flag antibody. The positions of molecular mass markers are indicated at the right.

Fig. 7 | Effect of the KCNQ1-*sid*_{Q3}/KCNQ2 interaction on surface expression. A, Surface expression of the HA-tagged KCNQ2 (normalized to the value for KCNQ2(HA) + KCNQ3 co-injection); B, surface expression of the HA-tagged KCNQ1-*sid*_{Q3} construct (normalized to the mean value for KCNQ1-*sid*_{Q3}(HA)). Oocytes were (co-)injected with KCNQ cRNAs as indicated. Values are means ± s.e. ($n \geq 7$ oocytes from three frogs). Oocytes expressing untagged KCNQ1 served as a background control.

as was observed for HA-tagged KCNQ3. If we assume that single channel properties of channels containing KCNQ1-*sid*_{Q3} do not differ significantly from those of homomeric channels, our surface expression data correlate with the current measurements. Schwake et al. (2000) found that neither single-channel conductances nor open probabilities of KCNQ2/KCNQ3 heteromers were increased compared with homomeric channels (KCNQ2/KCNQ3, 16 pS; KCNQ2, 18 pS; KCNQ3, 7 pS), a conclusion that was also drawn by Selyanko et al. (2001). If the same was true for KCNQ2/KCNQ1-*sid*_{Q3} heteromeric channels, the lower single-channel conductance of KCNQ1 (1.8 pS (Pusch, 1998) or 4 pS (Sesti & Goldstein, 1998)), compared with 7.4 pS reported for KCNQ3 (Schwake et al., 2000) could well account for the smaller current increase found for co-expression of KCNQ1-*sid*_{Q3} with KCNQ2.

In summary, we conclude that the *si* domain, which encompasses the previously described highly conserved A-domain and a domain important for formation of functional KCNQ1 homomers, suffices to determine the subunit specificity of KCNQ channel assembly.

METHODS

Complementary DNA constructs. Starting from KCNQ cDNAs subcloned into expression vector pTLN, the KCNQ1/KCNQ3 chimeras (see Fig. 1), the HA-tagged KCNQ1 and pore mutants KCNQ1(G314S) and KCNQ3(G318S) were constructed by recombinant polymerase chain reaction and verified by sequencing. The HA-tagged KCNQ1 contained the amino acid sequence

SEHYPDVDPYAVTF (the HA epitope is shown in bold), inserted after amino acid Ala 149 within the extracellular S1–S2 loop. The HA-tagged KCNQ2 and KCNQ3 constructs were as described previously (Schwake et al., 2000). Flag-tagged KCNQ1 and KCNQ2 contained a Flag epitope at the N terminus.

Expression in *Xenopus laevis* oocytes. Individual stage V–VI oocytes were obtained from anaesthetized frogs and isolated by treatment with collagenase. Synthesis of cRNA was performed with the SP6 mMessage mMachine kit (Ambion). KCNQ cRNA (10 ng) was injected into oocytes (also for co-injection experiments, which contained a 1:1 cRNA mixture). For KCNE1 co-expression, 1 ng of KCNE1 cRNA was added. After injection, oocytes were kept at 17 °C in MBS solution (88 mM NaCl, 2.4 mM NaHCO₃, 1 mM KCl, 0.41 mM CaCl₂, 0.33 mM Ca(NO₃)₂, 0.82 mM MgSO₄, 10 mM HEPES, pH 7.6).

Electrophysiology. Two or three days after injection, currents were measured in ND96 buffer (96 mM NaCl, 1 mM MgCl₂, 0.2 mM CaCl₂, 5 mM HEPES, pH 7.4) at room temperature (21 °C) in two-electrode voltage clamp recordings with a TurboTEC 10C amplifier (NPI Electronic) and pClamp8 software (Axon Instruments). Voltage protocol for current recordings, unless stated differently: from a holding potential of –70 mV, cells were pulsed for 2.5 s to voltages between –80 mV and +60 mV in steps of 20 mV, followed by a 2-s test pulse to –40 mV.

Surface expression experiments. Analysis of the surface expression of HA-tagged KCNQ constructs was as described (Zerangue et al., 1999). Oocytes were placed for 30 min in BS blocking solution (ND96 buffer plus 1% BSA), incubated for 1 h with rat monoclonal anti-HA antibody 3F10 (Roche Molecular Biochemicals) diluted to 1 µg ml^{–1} in BS solution, washed three times and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (goat-anti-rat Fab fragments; Jackson) in BS solution, followed by three washes each, first in BS solution, then in ND96 buffer. All incubation and washing steps were performed on ice. Surface expression was quantified by placing individual oocytes in 50 µl of SuperSignal ELISA Femto Maximum Sensitivity Substrate solution (Pierce); luminescence was measured in a TD 20/20 luminometer (Turner Designs).

Co-immunoprecipitation and western blot analysis. Oocytes were homogenized in ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 7.4 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4) containing 1 × Complete and 4 mM Pefablock (Roche), and yolk platelets were removed by three low-speed centrifugation steps. Membranes were pelleted at 100,000g (0.5 h, 4 °C) and resuspended in lysis buffer (120 mM NaCl, 5 mM dithiothreitol, 1 mM EGTA, 0.5% Nonidet P40, 10% glycerol, 4 mM Pefablock, 1 × Complete, 50 mM Tris-HCl, pH 8.0). Proteins were immunoprecipitated with the 3F10 anti-HA monoclonal antibody (Roche) for 4.5 h and with protein G–Sepharose (Roche) for another 0.5 h at 4 °C. After five washes with lysis buffer, immunoprecipitates were released at 60 °C in SDS sample buffer for 5 min and separated on 10% SDS–polyacrylamide gels. Immunodetection used primary mouse anti-Flag monoclonal M2 (Sigma) and secondary HRP-conjugated goat anti-mouse (Jackson) antibodies. Reacting proteins were detected with the Renaissance reagent (NEN) and photographic films.

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