

Molecular Basis for Interactions of G Protein $\beta\gamma$ Subunits with Effectors

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Both the α and $\beta\gamma$ subunits of heterotrimeric guanine nucleotide-binding proteins (G proteins) communicate signals from receptors to effectors. $G\beta\gamma$ subunits can regulate a diverse array of effectors, including ion channels and enzymes. $G\alpha$ subunits bound to guanine diphosphate ($G\alpha$ -GDP) inhibit signal transduction through $G\beta\gamma$ subunits, suggesting a common interface on $G\beta\gamma$ subunits for $G\alpha$ binding and effector interaction. The molecular basis for interaction of $G\beta\gamma$ with effectors was characterized by mutational analysis of $G\beta$ residues that make contact with $G\alpha$ -GDP. Analysis of the ability of these mutants to regulate the activity of calcium and potassium channels, adenylyl cyclase 2, phospholipase C- β 2, and β -adrenergic receptor kinase revealed the $G\beta$ residues required for activation of each effector and provides evidence for partially overlapping domains on $G\beta$ for regulation of these effectors. This organization of interaction regions on $G\beta$ for different effectors and $G\alpha$ explains why subunit dissociation is crucial for signal transmission through $G\beta\gamma$ subunits.

Upon receptor activation, G proteins dissociate into free $G\alpha$ and $G\beta\gamma$ subunits that can activate various effectors (1). Effector proteins of the $G\beta\gamma$ complex include phospholipases (2), adenylyl cyclases (3), ion channels (4), G protein-coupled receptor kinases (5) and phosphoinositide 3-kinases (6). Other potential $G\beta\gamma$ effectors include dynamin I and the nonreceptor protein tyrosine kinases Btk and Tsk (7). GDP-bound $G\alpha$ subunits ($G\alpha$ -GDP) can compete with $G\beta\gamma$ effectors and deactivate $G\beta\gamma$ -dependent signaling, suggesting that $G\beta\gamma$ may use a common binding surface for interaction with $G\alpha$ and with its diverse effectors. Two regions on $G\beta\gamma$ that interact with $G\alpha$ have been defined by the crystal structures of heterotrimeric $G\alpha\beta\gamma$ (8), the switch interface ($G\beta$ residues 57, 59, 98, 99, 101, 117, 119, 143, 186, 228, and 332) and the NH_2 -terminal interface ($G\beta$ residues 55, 78, 80

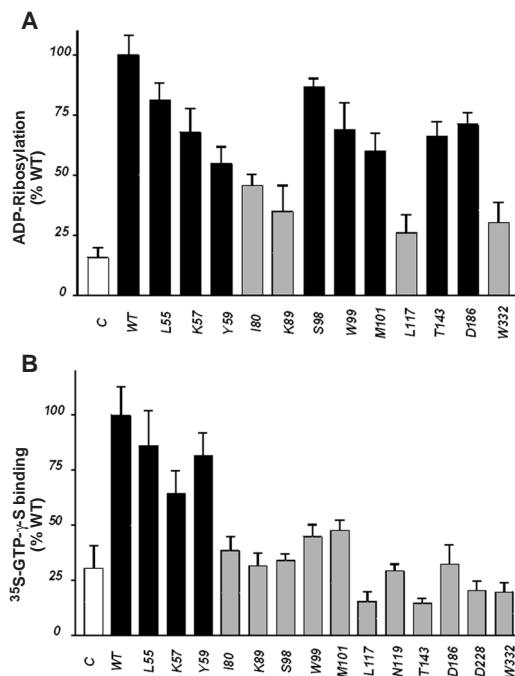
and 89). Each of these residues on retinal $G\beta$ ($G\beta$ 1) was substituted with alanine, and each $G\beta$ 1 mutant was expressed with either $G\gamma$ 1 or $G\gamma$ 2, two isoforms of the $G\gamma$ subunit. All mutated $G\beta$ 1 dimers were folded properly, were post-translationally modified appropriately, and were expressed at similar amounts as in the wild type (9). The $G\beta\gamma$ mutants were tested for their ability to assemble into heterotrimers with $G\alpha$, to be activated by rhodopsin,

and to interact with $G\beta\gamma$ downstream signaling partners: β -adrenergic receptor kinase (β ARK), phospholipase C- β 2 (PLC- β 2), adenylyl cyclase 2 (AC2), muscarinic potassium channel (GIRK1/GIRK4), and the calcium channel α 1B subunit (CC α 1B).

To determine whether purified $G\beta$ 1 $H_{6\gamma}$ 1 mutants could form heterotrimers, we measured the ability of the $G\beta\gamma$ mutants to facilitate pertussis toxin-catalyzed adenosine diphosphate (ADP) ribosylation of transducin $G\alpha$ -GDP ($G\alpha$) (10). All mutants could support some level of ADP ribosylation, although $G\beta$ mutants Ile⁸⁰ \rightarrow Ala⁸⁰ (I80A), K89A, L117A, and W332A (11) showed reduced ability to form heterotrimers (Fig. 1A).

Because $G\beta\gamma$ is essential for functional heterotrimer interaction with activated receptors that catalyze the exchange of GDP for guanosine triphosphate (GTP) on the $G\alpha$ subunit, we also measured the ability of the $G\beta$ mutants to support light-activated rhodopsin-catalyzed nucleotide exchange on the α subunit of transducin ($G\alpha$) (12). All switch interface mutants (except K57A and Y59A) and the NH_2 -terminal interface mutants I80A and K89A were defective in formation of functional heterotrimers (Fig. 1B). Some $G\beta$ mutants were impaired in both assays, indicating that residues 80, 89, 117, and 332 of $G\beta$ are the major determinants of binding to $G\alpha$. The switch interface mutants (S98A, W99A, M101A, N143A, and D186A) were normal in heterotrimer assembly, but were impaired functionally in supporting receptor-catalyzed nucleotide ex-

Fig. 1. Effects of $G\beta$ 1 $H_{6\gamma}$ 1 on heterotrimer assembly and receptor interaction. The data are the normalized percentage of wild-type (WT) recombinant $G\beta\gamma$ activity. **(A)** The ability of recombinant $G\beta$ 1 $H_{6\gamma}$ 1 and $G\beta$ 1 mutants to assemble into heterotrimers with $G\alpha$ was determined by testing whether pertussis toxin could ADP-ribosylate $G\alpha$ with [³²P]nicotinamide adenine dinucleotide (10). The $G\beta$ residue mutated to alanine is indicated by a number beneath each bar in the figure. Clear bar (C) represents the basal amount of ADP-ribosylation of $G\alpha$ that occurred in absence of $G\beta\gamma$. **(B)** The ability of recombinant $G\beta$ 1 $H_{6\gamma}$ 1 and $G\beta$ 1 mutants to bind $G\alpha$ and interact with rhodopsin was determined by the amount of [³⁵S]GTP- γ -S binding catalyzed by light-activated rhodopsin (12). Clear bar (C) is the basal amount of [³⁵S]GTP- γ -S binding to $G\alpha$ in the presence of urea-washed rod outer segment membranes (50 nM) without added $G\beta\gamma$. The data represent the mean \pm SEM of duplicate determinations in three independent experiments. Alanine mutants that have a distinguishable activity from the wild type are indicated by gray bars; those with activity similar to the wild type are indicated by black bars (11).



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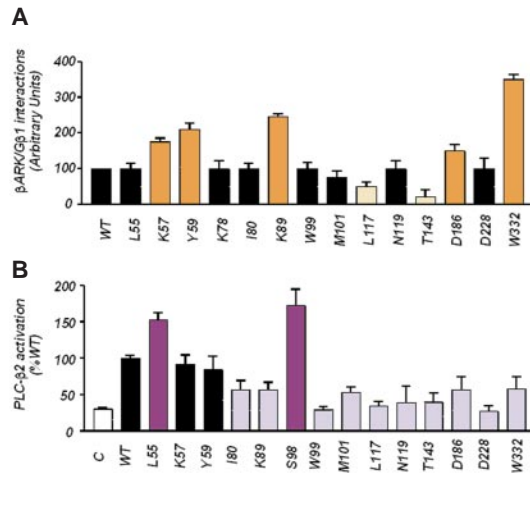
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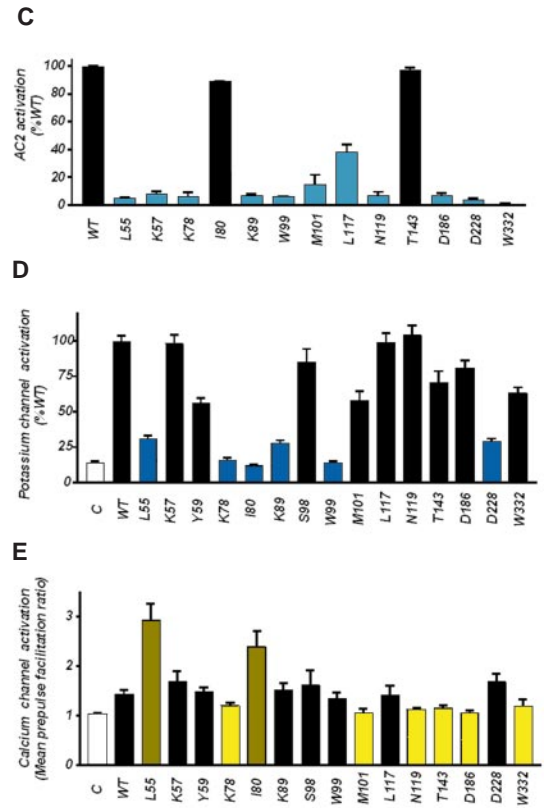
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Fig. 2. G β γ -dependent interactions with β ARK, PLC- β 2, AC2, muscarinic potassium channel, and CC α 1B-containing calcium channel. **(A)** The amount of G β 1 present in β ARK immune complexes was detected as described (14). Light orange bars show those mutations that decrease G β γ / β ARK interaction, while dark orange bars show mutations that increase the interaction. The data contained in the bar graph represent duplicate determinations in two independent experiments. **(B)** G β 1 γ 1-dependent activation of PLC- β 2 was determined as described (17).



Clear bar (C) represents the basal PLC- β 2 activity in the absence of G β γ . Light purple bars show those mutations that decrease G β γ -mediated PLC β 2 activation, while dark purple bars indicate mutations that determine enhanced activation. The data are presented as the normalized percentage of wild-type recombinant G β γ activity and represent the mean \pm SEM of duplicate determinations in three independent experiments. **(C)** G β 1 γ 2-dependent activation of AC2 was determined by reconstituting membranes as described (18). Data represent duplicate determinations from two independent experiments. **(D)** G β 1 γ 2-dependent activation of GIRK potassium channel was determined as described (19). Protein immunoblotting showed that all mutants were expressed in equal amounts. Clear bar (C) represents control oocytes injected with GIRK1, GIRK4, and G γ 2 RNAs. **(E)** G β 1 γ 2-dependent inhibition of CC α 1B calcium channels was determined as described (21). Protein immunoblotting revealed that all mutants were expressed in similar amounts. The amount of G β γ -dependent inhibition was calculated as a ratio of the mean prepulse facilitation (MPF) in either the absence or presence of G β γ . MPF is the statistically averaged relief of the channel inhibition by use of a large depolarizing prepulse. Clear bar (C) represents channel activity in absence of G β γ . The light yellow bars indicate the G β 1 mutations that decrease channel modulation and lead to calcium currents that are statistically indistinguishable from the basal calcium current ($P < 0.1$ for K78A and W332A mutants and $P < 0.01$ for M101A, N119A, T143A, and D186A mutants). The dark yellow bars indicate G β 1 mutants that have increased inhibitory activity ($P < 0.001$ for L55A mutant and $P < 0.01$ for I80A mutant).



change on G α . This observation indicates that G β γ may actively participate in receptor-catalyzed nucleotide exchange, rather than being simply a passive binding partner in receptor-G protein interactions.

G β γ mediates translocation of G protein-coupled receptor kinases from the cytosol to the membrane, in order that these kinases can phosphorylate activated G protein-coupled receptors and initiate receptor internalization (13). The G β mutants varied in their ability to associate with β ARK1 (Fig. 2A) (14). Alanine mutations at G β residues 117 and 143 resulted in decreased binding to β ARK1. In contrast, alanine mutations at G β residues 57, 59, 89, 186, and 332 of G β led to increased binding to β ARK1. The mutations that resulted in decreased binding are found on the left side of the G β surface (Fig. 3) and likely form the β ARK binding interface, whereas those mutations that led to increased binding were clustered together at the middle of the structure (G β residues 57, 59, and 332) or are at the right side of the surface (G β residue 89).

G β γ is an important modulator of various isoforms of phospholipase C- β (2, 15) and adenylyl cyclase (16), effectors that

regulate intracellular concentration of second messengers inositol 1,4,5-triphosphate and cyclic adenosine 3',5'-monophosphate. The ability of the G β γ mutants to stimulate the activity of PLC- β 2 was determined by quantitating the amount of inositol 1,4,5-triphosphate produced by the purified enzyme in the presence of the G β γ mutants (17). Some 13 of the 15 G α -interacting residues of G β we tested were important for G β γ -dependent activation of PLC- β 2, suggesting that G α and PLC- β binding regions on G β are overlapping (Fig. 2B). Mutants W99A and D228A no longer activated PLC- β 2 and mutants I80A, K89A, M101A, L117A, N119A, T143A, D186A, and W332A were less effective than wild-type G β γ . Mutants L55A and S98A activated PLC- β 2 to a greater extent than wild-type G β γ . These residues are circled in magenta on the G β γ surface (Fig. 3). The effects of the G β γ mutants on AC2 activation were determined in vitro (18) in the presence of constitutively activated G α that has glutamine at residue 227 mutated to leucine (Q227L). All the Ala mutations of G β residues, except I80A and T143A, had decreased ability to activate AC2 (Fig. 2C); their locations on the G β γ structure are

indicated in teal (Fig. 3).

We also measured K $^{+}$ currents in *Xenopus laevis* oocytes injected with RNAs for GIRK1/GIRK4 and G β γ mutants (19). The ability of G β γ to increase conductance through the muscarinic potassium channel GIRK1/GIRK4 was disrupted by alanine mutations at G β residues 55, 78, 80, 89, 99, and 228 (Fig. 2D). All these G β residues except W99 and D228 cluster within the NH $_2$ -terminal interface of G β (Fig. 3; blue lines).

G β γ inhibits the activity of certain calcium channels (20). We measured the ability of G β γ mutants to inhibit the conductance of Ca $^{2+}$ channels in HEK 293 cells expressing CC α 1B-containing Ca $^{2+}$ channels and G β γ mutants (21). Alanine mutations of G β residues 55 and 80, which lie close together at the top of G β , had enhanced ability to inhibit current through CC α 1B-containing Ca $^{2+}$ channels (Fig. 2E). Alanine mutations of G β residues 78, 101, 119, 143, 186, and 332 were no longer able to inhibit current through calcium channels.

Our results demonstrate that many of the G α -interacting residues of G β are important in interactions between G β γ and

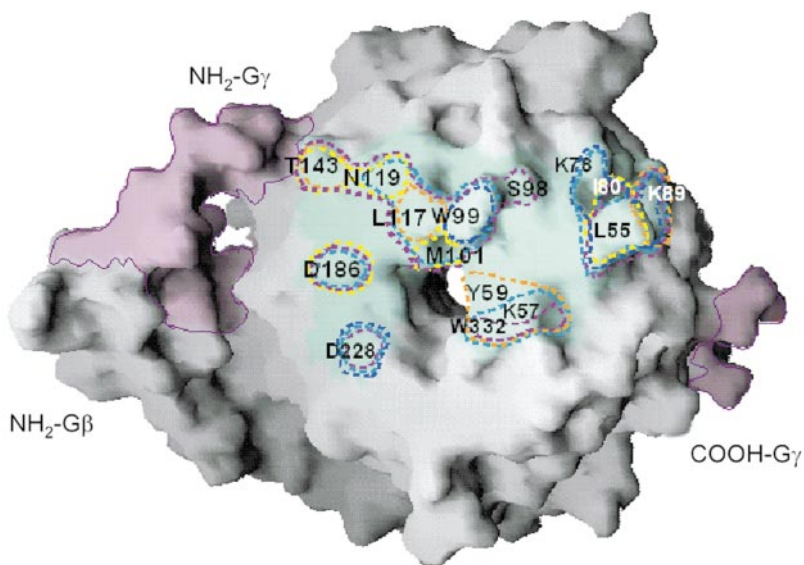


Fig. 3. A schematic representation of the regions of G β involved in interactions with effectors and G α subunit. The crystal coordinates of G β 1 γ 1 [Protein Databank entry 1tbg; (8, 26)] were used to generate a surface model of the dimer in GRASP. G β is gray, and G γ is pink. The pale green surface is the area on G β that is covered by G α in the G protein heterotrimer crystal structure. The effector-interacting residues on G β are circled with a different color for each effector: orange, β ARK; magenta, PLC- β 2; teal, AC2; blue, potassium channel; and yellow, calcium channel. G α -GDP, when bound to G β γ , covers all these distinct yet partially overlapping effector interaction regions on G β and, thus, blocks G β γ regulation of all the effectors. This figure and other more detailed figures showing residues interacting with individual effectors can be viewed at www.sciencemag.org/feature/data/976104.shl

its signaling partners, and show the functional importance of individual amino acids in the signal transfer from G β γ to effector activation. Alanine mutation of these G β residues may increase, decrease, or abolish G β γ -dependent interactions. It was unexpected to find G β γ mutants that are better than the wild type at stimulating the activity of PLC- β 2 and inhibiting Ca²⁺ channels. One possible reason for such “gain-of-function” mutations is that turn-off of G β γ -mediated signaling requires G α -GDP competition with effectors. The normal side chains at these positions may be destabilizing to effector interaction resulting in a lower affinity in order to allow reassembly of heterotrimeric G α β γ .

Each signaling partner for G β relies on a different subset of G β residues for its interaction and hence, creates a set of unique “footprints” on G β (Fig. 3). These results are consistent with studies that have suggested a common effector binding surface on G β γ located near the region of residues 70 to 145 of G β (22). These data raise an interesting issue of how G proteins and their effectors are oriented with respect to the membrane and whether their orientations change during subunit dissociation and activation (8, 23). The G α -binding surface on G β γ may not be the only region of effector interaction. Other G β γ regions of effector interactions that have been implicated are the coiled-coil interface at the

NH₂-termini of G β and G γ (24) and the COOH-terminal region of G β (25).

The alanine mutations of G α -interacting G β residues provide an initial framework to determine how G β γ subunits interact with and regulate so many different effectors that have so little structural similarity. Our studies show that the effector interaction regions are clustered on G β such that they partially overlap one another (Fig. 3). This mode of clustering allows for one key regulator (G α) to regulate G β γ signal transmission to multiple effectors.

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- viruses, detailed protocols for recombinant protein expression in Sf9 insect cells, and detailed protocols for purification of G β γ complexes are described at www.sciencemag.org/feature/data/976104.shl#9. The recombinant wild-type G β γ was equivalent to native G β γ in the assays used in our study. All mutated G β 1 subunits assembled properly with H₆G γ 1 subunits as judged by the trypsin protection assay. We determined, with mass spectrometry, that the recombinant G β γ dimers tested were properly modified by isoprenylation and carboxymethylation.
- The pertussis toxin-catalyzed ADP ribosylation assay was done essentially as described [T. Katada *et al.*, *Methods Enzymol.* **237**, 131 (1994)]. Details of modifications are provided at www.sciencemag.org/feature/data/976104.shl#10.
- Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Distinct WNT Pathways Regulating AER Formation and Dorsoventral Polarity in the Chick Limb Bud

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The apical ectodermal ridge (AER) is an essential structure for vertebrate limb development. *Wnt3a* is expressed during the induction of the chick AER, and misexpression of *Wnt3a* induces ectopic expression of AER-specific genes in the limb ectoderm. The genes β -catenin and *Lef1* can mimic the effect of *Wnt3a*, and blocking the intrinsic *Lef1* activity disrupts AER formation. Hence, *Wnt3a* functions in AER formation through the β -catenin/LEF1 pathway. In contrast, neither β -catenin nor *Lef1* affects the *Wnt7a*-regulated dorsoventral polarity of the limb. Thus, two related *Wnt* genes elicit distinct responses in the same tissues by using different intracellular pathways.

The *Wnt* gene family encodes a group of signaling molecules that are implicated in numerous aspects of morphogenesis in both vertebrates and invertebrates. Several chick *Wnt* genes are expressed in a specialized epithelial structure running along the distal margin of the limb bud, called the apical ectodermal ridge (AER), which is essential for limb morphogenesis (1, 2). *Wnt3a* is the first of these genes to be expressed in the limb. We therefore examined the spatio-temporal pattern of expression of *Wnt3a* in developing limb buds with respect to that of *Fgf8*, the earliest known AER marker during chick development (3, 4) (Fig. 1, A through D) (5).

Wnt3a transcripts are detected before

Fgf8 transcripts in the limb field ectoderm but not in the flank outside the limb fields. Subsequently, *Wnt3a* expression is up-regulated in the ectoderm cells near the dorsoventral (DV) border. *Fgf8* expression is initiated and then up-regulated within the region of high *Wnt3a* expression during AER formation. From stage 20 on, *Wnt3a* and *Fgf8* expression are confined primarily to the mature AER. Thus, *Wnt3a* expression appears to presage *Fgf8* expression and AER formation.

To verify the epistatic relationship between *Wnt3a* and *Fgf8* that is suggested by the expression data, we ectopically delivered each factor to developing limb buds. We misexpressed *Wnt3a* in the limb ectoderm using a replication-competent retroviral vector and assayed for the expression patterns of the various AER markers (6). Misexpression of *Wnt3a* induced ectopic expression of AER-specific genes, including *Bmp2*, *Fgf4*, and *Fgf8*, in broad patchy domains in the ectoderm of nearly 100% of infected limbs (Fig. 1E) (5). However, *Wnt3a* expression was not induced in the ectoderm by either fibroblast growth factor 4 (FGF4) protein or *Fgf8*-virus (5). This suggests that *Wnt3a* acts upstream of FGFs in establishing AER gene expression.

In addition to its effect on AER gene expression, *Wnt3a* misexpression occasion-

ally led to disruption of the AER or to formation of an ectopic AER extending ventrally, or both (Fig. 1, E and F). These morphological effects on the AER are reminiscent of those seen after misexpression of *Radical fringe* (7). We therefore examined *Radical fringe* expression and found that it was ectopically expressed in *Wnt3a*-infected limbs (8). Disruption of the AER morphology was only seen in a subset of *Wnt3a*-infected limb buds, which is consistent with the finding that *Radical fringe* only affects AER formation when it is misexpressed at the earliest stages of limb development (7).

The FGFs produced in the AER are responsible for maintaining the proliferative state of the undifferentiated mesoderm at the distal tip of the limb bud, the progress zone (PZ) (1). To verify that the FGFs induced in the limb bud ectoderm by *Wnt3a* are functional signals, we examined the expression of several PZ markers: *Fgf10* (9), *Msx1* (10), *Nmyc* (11), and *Slug* (12). Equivalent results were obtained with each of these markers (Fig. 2) (8). When the AERs were removed from experimental limb buds, expression of the PZ markers was rapidly lost (Fig. 2, C and D). Application of *Wnt3a*-expressing cells to the AER-deprived limbs induced *Fgf8* expression and restored expression of the PZ markers (Fig. 2, E and F). To show that this response was due to the ectopic expression of FGFs and not to a direct action of *Wnt3a* itself, we removed the adjacent distal ectoderm as well as the AER so that the *Wnt3a* cells could not induce ectodermal *Fgf8* expression (Fig. 2G). Under these conditions, *Wnt3a* induced little or no expression of the PZ genes (Fig. 2H). The maintenance of the PZ is critical for outgrowth of the limb bud. The long-term effects of virally mediated *Wnt3a* misexpression, and consequent ectopic FGF production by the ectoderm, included some cases in which extra outgrowth formed digitlike structures (5). *Wnt3a* thus appears to influence both morphological AER formation and induction of AER-specific genes in the early limb bud.

The members of the vertebrate *Wnt* gene family have been categorized by their relative ability to transform murine mammary epithelial cells (13). A similar classification can be made on the basis of the ability to

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