

Wnt Signaling

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I. Introduction

The *Wnt* gene family encodes growth factors that were originally identified by their ability to induce mammary gland tumors in mouse model systems. The *Wnt-1* gene (originally *int-1*) was identified as a frequent target for insertional activation by mouse mammary tumor virus (MMTV), a virus known to cause mammary gland tumors in mice. Inappropriate expression of either *Wnt-1* or *Wnt-3* leads to epithelial proliferation in the mammary gland and eventually tumors. These findings originally placed *Wnt* genes in the family of oncogenes involved in tumor development.

Most *Wnt* genes have been isolated by cloning genes homologous to *Wnt-1* and they encode cysteine-rich, secretory glycoproteins from 350-380 amino acids. Studies of the biochemical properties of Wnt proteins have largely been carried out with cultured cells programmed to express exogenous Wnt proteins. These studies suggest that Wnt proteins act as diffusible secreted factors that are tightly associated to extracellular proteins, making the levels of extracellular, soluble Wnt proteins low. Analysis of wingless (*wg*) protein function, the *Drosophila* homologue of *Wnt-1*, suggests that it acts in a paracrine fashion. These observations have led to the model that Wnt proteins are local-acting factors, that is, they function to signal cells adjacent to or near the site of Wnt production. Biologically active, soluble *wg* or *Wnt-1* proteins have been generated as conditioned media preparations, yet despite such progress, purification of active Wnt or *wg* proteins has yet to be accomplished.

The normal functions of *Wnt* genes have been analyzed in organisms amenable to genetic analysis of early development. The *Wnt-1* orthologue in *Drosophila* is the segment polarity gene *wingless*. Genetic and biochemical analyses suggest that the *wg* protein functions as a local-acting, secreted factor that triggers a cascade of molecular events that assist in directing body plan organization of the *Drosophila* embryo and organization of structures such as the limbs and wings. In the frog, *Xenopus laevis*, several different *Wnt* genes have been shown to contribute to the experimental induction of dorsal mesoderm tissue and subsequent establishment of the body axis. This induction can lead to complete duplication of the body axis.

II. Wnt signaling pathway

The nature of the signaling events triggered by Wnt proteins has become apparent. Genetic and biochemical analysis of the *wg* signal transduction pathway in *Drosophila* embryos and cultured insect cells suggest a cascade of events distinct from any previously described signal transduction pathway. The elements of the pathway defined genetically in *Drosophila* have been conserved in vertebrates, such as mouse or man. An elementary model for the Wnt signaling pathway is schematized in Figure 1.

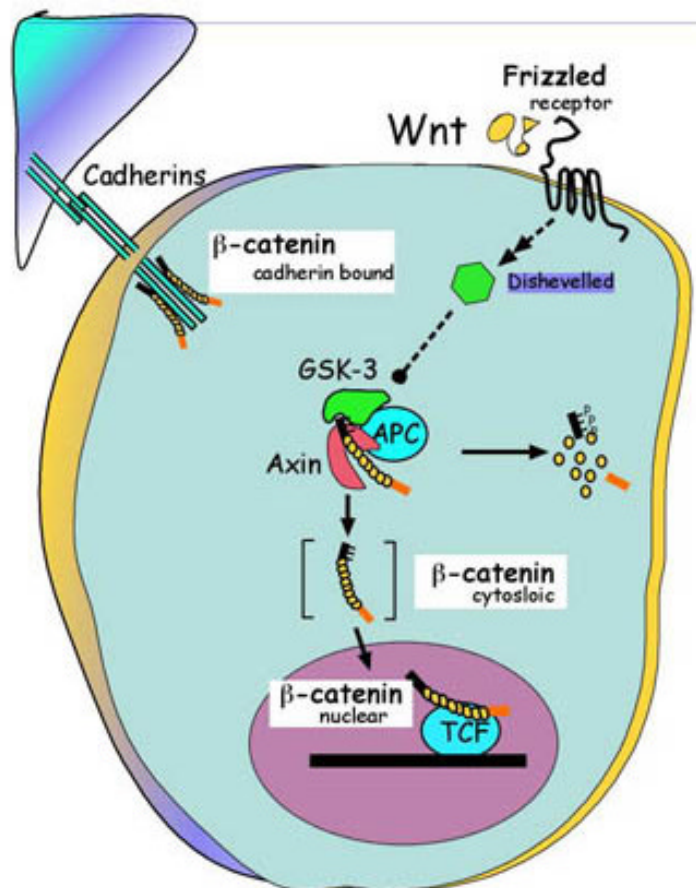


Figure 1. Wnt Signaling Pathway. (See text for details and description of components of the pathway)

Wnts interact with the Frizzled cell surface receptor. Frizzled proteins contain a conserved amino-terminal cysteine-rich domain and seven putative transmembrane segments. Genetic analysis in *Drosophila* shows that Frizzleds are required for Wnt signaling and biochemical studies have established that Frizzled proteins can bind Wnts. Within the target cell, the cytosolic Dishevelled protein is the first known intracellular component in Wnt-mediated signaling. However, the specific function of Dishevelled has not yet been established. Downstream of Dishevelled is the serine/threonine protein kinase Glycogen Synthase Kinase 3 (GSK-3), whose activity must be suppressed to transmit Wnt signals. In cells where Wnt/Frizzled signals are not on, GSK-3 continually phosphorylates β catenin. This phosphorylation marks catenin as a target for proteolytic degradation. Suppression of the GSK-3 kinase activity leads to dephosphorylation and increased stability of cytosolic β catenin protein.

Several proteins have been proposed to form a large complex within the cytosol whose goal is to recognize the phosphorylated form of β -catenin and target the protein for destruction. A notable component of this complex is the tumor suppressor Adenomatous Polyposis Coli or APC. APC likely mediates the targeting of β -catenin to the ubiquitin-mediated proteolysis pathway. Another protein in this complex is Axin. Axin is a protein structurally similar to RGS protein (Regulators of G-Protein Signaling) and to Dishevelled. Disruption of this gene in the mouse germ

line causes embryonic axis duplication, a response similar to the effect of excess Wnt in *Xenopus* embryos, which leads to axis duplication. This remarkable finding in mouse studies suggests that Axin functions as a negative regulator of Wnt signaling, a proposal born out after analysis of Axin function in several organisms from flies to frogs. When the targeting activity of this GSK-3/APC/Axin complex is suppressed the levels of cytosolic β -catenin increase dramatically. This increase in the steady state levels of β -catenin promotes the transit of β -catenin to the nucleus and the formation of a complex with the TCF/LEF-1 family of transcription factor.

β -catenin/TCF complexes form in the nucleus and directly regulate the expression of target genes of the Wnt signaling pathway.

III. β -catenin; the central player in signal transduction

Most of the identified proteins within the Wnt signaling pathway are involved in regulating the activity and levels of β -catenin. Thus, β -catenin functions as the central mediator of Wnt signaling and is the key protein responsible for conversion from the “signal off” state to “signal on”. Catenins exist in three isoforms, α , β , and γ , which form a complex with cadherins. This complex resides at the cytoplasmic tail of the membrane-spanning cadherins (Figure 1). Cadherin-catenin complexes are essential for cell-cell adhesion. Within cells, the majority of β -catenin is found in association with cadherins at the membrane. The signaling form of β -catenin is not associated with cadherins and resides in the cytosol or nucleus.

To focus on β -catenin as a signal transduction protein, one can analyze the fate of β -catenin. Cytosolic β -catenin responds to Wnt signals, whereas cadherin-bound β -catenin does not. Operationally, this is accomplished by evaluating the membrane-bound and cytosolic forms of β -catenin. Cells to be analyzed are Dounce Homogenized and high speed centrifugation is used to separate a supernatant fraction (cytosolic) and a pellet (membranous). The resultant fractions are then analyzed by immunoblotting using anti β -catenin antibodies. To date, this is one of the most sensitive assays for Wnt-signaling.

To study the mechanism of action of β -catenin and Wnt signaling in colon cancer cells, we established that β -catenin is constitutively stabilized in cells with APC or β -catenin mutations (see section IV for description of mutations). DLD-1 is a colon cancer cell line that expresses a truncated APC and displays increased TCF transcriptional activity. HCT116 is a colon cancer cell line that expresses mutated β -catenin that lacks Ser-45 and also displays increased TCF transcriptional activity. When analyzed for cytosolic β -catenin levels in HCT116 and DLD-1 we found that in both colon cancer cells lines β -catenin was constitutively stabilized in the cytosol and Wnt-1 expression did not lead to further stability of β -catenin (Figure 2). As a positive control, the β -catenin response in the colon cancer lines was compared to the response found in Rat-1 fibroblasts. Normal Rat-1 fibroblasts have low cytosolic levels of β -catenin and thus are in the “signal off” state whereas Wnt-1 stimulated Rat-1 cells have elevated levels of cytosolic β -catenin, or “signal on.”

IV. Human cancers and APC/ β -catenin

Several genes involved in Wnt signaling act as oncogenes or tumor suppressor genes. The *APC* gene functions as a tumor suppressor, that is, it encodes a protein that normally functions as a growth suppressor in colon epithelium. *APC* was originally implicated in the Wnt signaling pathway based on its ability to associate with β -catenin and GSK-3. Earlier work established that loss of *APC* is correlated with cancer progression in the colon. This loss can be acquired in an individual through inheritance of a mutated *APC* gene. Such individuals have Familial Adenomatous Polyposis (FAP) syndrome manifested by numerous colonic polyps that develop into carcinoma. Loss of *APC* can also be acquired through somatic mutation of the gene, also leading to colon cancer development. Mutations of *APC* almost always lead to the expression of a truncated *APC* protein missing much of the domain of *APC* that has been defined as necessary for binding to β -catenin.

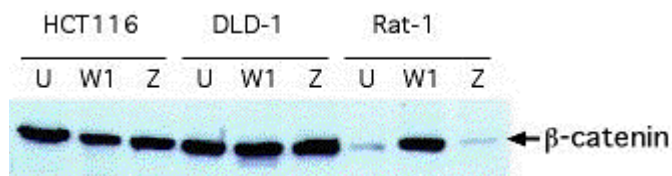


Figure 2. Levels of Cytosolic β -catenin in Colon Cancer Cell Lines. DLD-1 is a human colon cancer cell line that expresses a truncated *APC*. HCT116 is a human colon cancer cell line that expresses mutated β -catenin. HCT116, DLD-1, or Rat-1 cells were either mock infected (Ctr) or infected with either Adenovirus vectors expressing Wnt-1 (W1) or LacZ (Z). Cells were Dounce Homogenization and centrifugation was used to prepare a high-speed supernatant, designated cytosolic fraction. Antibodies against β -catenin were used to generate immune-complexes from the cytosolic fraction and the complexes were analyzed by immunoblotting for β -catenin.

The link between loss of *APC* and activation of Wnt signaling by excess β -catenin is compelling. Colon carcinoma cells devoid of *APC* contain elevated β -catenin levels (Figure 2) and a β -catenin-TCF complex that is constitutively active. Thus, constitutive transcription of TCF target genes can be caused by loss of *APC* function suggesting that activation of the Wnt signaling pathway is a key event in the transformation of colonic epithelium.

Genetic defects that result in deregulation of β -catenin play a role in melanoma progression. Abnormally high levels of β -catenin due to missplicing or missense mutations of the *β -catenin* gene were detected in several human melanoma cell lines. Other melanoma lines are missing *APC* or contain structurally altered *APC* proteins. These alterations are associated with constitutive activation of β -catenin/TCF transcription complexes.

Mutation of the *β -catenin* gene has been reported for a variety of other human tumors. Most of the identified mutations affect one of the putative GSK-3 phosphorylation sites of β -catenin (S33, S37, T41 and S45 amino acid residues in human β -catenin). Mutations in the *β -catenin* gene have been found in colorectal adenoma, colorectal carcinoma, melanoma, endometrial carcinoma, gastric carcinoma, hepatocellular carcinoma, malignant fibrous histiocytoma, medulloblastoma, ovarian carcinoma, pilomatricoma, prostate cancer, synovial

sarcoma, and uterine cancer. β -catenin is thus a target for oncogenic mutations in a wide variety of human tissues.

APC mutations are most highly correlated with colon cancer and such mutations are less frequently found in other tumor types. Interestingly, although Wnt signaling promotes mammary tumors in mouse models, mutations in *APC* or β -catenin genes in human breast cancer are rare. Mutation of *APC* is also found in another human tumor type, aggressive fibromatosis.

V. Oncoprotein pathways that intersect Wnt signaling

With the rapid progress in the molecular analysis of the Wnt signaling cascade, new players in the pathway have been identified. The variety of signaling components is described in several comprehensive reviews of the pathway and some are noted in Figure 3. Many of these new components participate in distinct signaling cascades known to function in cancer development. The Wnt/Frizzled cascade that regulates β -catenin also regulates other cellular targets. It is clear from *Drosophila* analysis that the *Drosophila* homologue of RhoA p21 GTPase has been genetically tied to Frizzled function. Vertebrate RhoA affects the JNK kinase signaling cascade, a pathway that regulates cellular proliferation. It is not clear how Wnt/Frizzled interaction ultimately regulates the RhoA/JNK cascade, but it is clear that the regulation is mediated through Dishevelled. The GSK-3 Binding Protein (GBP), a new component of the pathway, regulates GSK-3 activity and is related to the human *FRAT* gene (frequently rearranged in advanced T cell lymphomas). Analysis in *Xenopus* embryos of the Wnt cascade suggests a key role for Ca^{2+} signaling for some Wnt/Frizzled signaling functions. The Ser/Thr kinase Akt is involved in regulating cellular proliferation and can be regulated by the PTEN tumor suppressor. Akt also phosphorylates and suppresses GSK-3 activity but the link between PTEN/Akt and the Wnt signaling cascade has not been firmly established.

Other kinase cascades involved in cellular proliferation can target components of the Wnt cascade. The tyrosine kinases MET and Epidermal Growth Factor Receptor (EGFR) phosphorylate β -catenin on tyrosine residues. The consequences of this phosphorylation are unclear. The serine/threonine kinases, Casein kinase II and I ϵ , phosphorylate Dishevelled. It is well established that Casein kinase I ϵ directly affects Wnt signaling when analyzed in *C. elegans* and *Xenopus*. Components of the Mitogen Activated Protein Kinase (MAPK) cascade of *C. elegans*, NEK and TAL, have been implicated in phosphorylation and regulation of the TCF transcription factor. All these studies suggest that Wnt signaling is responsive to signals from other growth regulatory signaling cascades. The nature of the integration of such diverse signaling cascades in a cell is not understood but may be key to understanding the ultimate growth state of any given cell.

Wnt signaling can also transcriptionally activate cellular oncogenes. The *C-MYC* and *cyclin D1* genes are transcriptional targets of β -catenin/TCF. *C-MYC* expression is linked to a variety of human cancers and is essential for cell survival signals. Cyclin D1 promotes cell cycle progression and aberrant expression of cyclin D1 is also linked to human tumors. Current models propose that

Wnt/Frizzled signals may ultimately target several other growth-promoting gene products.

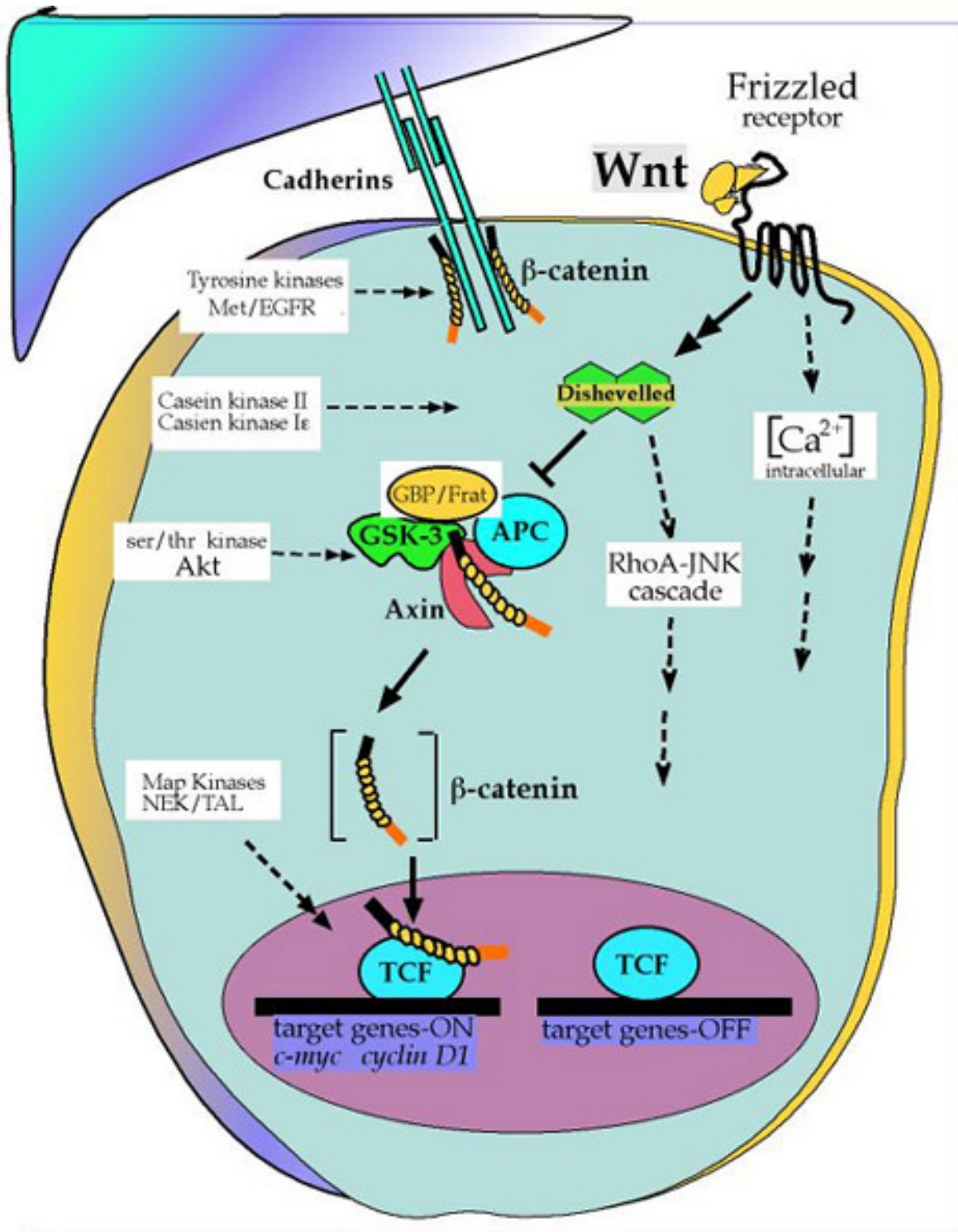


Figure 3. Intersection of Mitogenic Signaling Pathways with Wnt Signaling Cascade. Wnt signal OFF represents the pathway in the absence of Wnt. Wnt signal ON represents Wnt activated events leading to β-catenin stabilization (denoted by []) and activation of TCF target genes, *c-myc* and *cyclin D1*. Dotted arrows represent either intersecting pathways (Met, EGF Receptor, Casein Kinase II, Casein Kinase Iε, Akt, NEK, TAL) or parallel pathways (RhoA-JNK and intracellular Ca²⁺).

VI. Mouse models of Wnt function in oncogenesis.

Mouse models have illuminated the normal function of Wnt genes in many tissues. Although Wnts are clearly essential for induction and cell specification in both invertebrates and vertebrates, many of the mouse models point to a role for Wnts in promoting cellular proliferation. There are four illustrative target tissues where Wnt proliferative functions have been defined through experimental analysis using mouse: the colon, mammary gland, uterus, and the developing central nervous system.

Mouse models for loss of *Apc* have either arisen in nature, the *Apc* min mouse, or have been engineered using gene targeting strategies. In these models loss of *APC* leads to excess proliferation of intestinal epithelial cells and the development of numerous intestinal tumors. In intestinal epithelium a member of the TCF transcription family, TCF-4 likely mediates the Wnt response. Loss of Tcf-4 in mice leads to reduced proliferation of intestinal stem cells. It is likely that loss of *APC* leads to constitutive Tcf-4/ β -catenin activity promoting uncontrolled intestinal cell proliferation and intestinal tumors. Thus, in both mouse and man the Wnt signaling pathway via APC/ β -catenin/TCF is key to normal and abnormal proliferation of colonic epithelium.

In the mouse, the role of Wnts in mammary gland development and mammary oncogenesis is coming to light. Aberrant expression of either *Wnt-1* or *Wnt-4* does not solely convert the mammary ductal epithelium into carcinoma but induces hyperplasia and excess branching of the epithelial ducts. Other mutational events contribute further to tumor development. Studies *in vitro* with cultured mammary epithelial cells suggests that Wnts can act as both proliferative and morphogenic factors to promote epithelial tube formation, thus directing both growth and patterning. Endogenous *Wnt-2*, *Wnt-4*, *Wnt-5a*, *Wnt-5b*, *Wnt-6*, and *Wnt-7a* genes are expressed in the mammary gland during growth and differentiation (in virgin and pregnant mice) but they are not expressed in lactating glands, when the gland is no longer growing. Furthermore, the expression of several *Wnt* genes appears to be hormonally regulated. These two findings taken together suggest that regulated expression of *Wnt* gene products play a role in the normal expansion or differentiation of the mammary epithelium before lactation. Mutation of *APC* and *b-catenin* genes in human breast cancer is very rare, so the mouse models do not prove predictive for human breast cancer mutations involving Wnt signaling genes. However, as new components of the signaling pathway are defined and divergent pathways emerge, a role for Wnt signaling in human breast cancer may yet emerge.

Reproductive tract development and function is regulated by circulating steroid hormones such as estrogenic compounds that direct aspects of uterine gland formation and epithelial differentiation. The use of the synthetic estrogenic compound diethylstilbestrol (DES) in the 1950's on pregnant women to prevent possible miscarriage had the unexpected effect of inducing a range of gynecological problems including malformed reproductive tracts and a higher incidence of reproductive tract cancer in daughters of these women. Recent studies in the mouse have identified Wnts as signaling proteins with a role during uterine development and adult function.

For one member of the *Wnt* gene family, *Wnt-7a*, deregulation in mice in response to pre-natal exposure to DES, leads to uterine abnormalities reminiscent of the human condition. These malformations are also exhibited in a mouse lacking the *Wnt-7a* gene. These advances point to an important role for the Wnt gene family in various reproductive tract pathologies, including cancer.

In the central nervous system, Wnts promote the development of several structures or regions of the brain, including the cerebellum and hippocampus. In each of these regions, loss of the appropriate *Wnt* gene (*Wnt-1* for cerebellum and *Wnt-3a* for hippocampus) leads to reduced cellular proliferation. Wnt signaling is also essential for proliferation of neural crest and CNS progenitor cells.

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