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ESSENTIALS This chapter outlines the general principles of intracellular signalling. Focusing on cell surface receptors, the requirements for effective transmission of information across the plasma membrane are

outlined. The principal mechanisms utilized in mammalian signal transduction are described. For each, the pathological consequences of aberrant signalling and means by which pathways can be pharmacologically targeted are described in molecular terms. Intracellular signalling pathways permit the transmission and integration of information within cells. Mammalian receptor signalling relies on only a small number of distinct molecular processes which interact to determine cellular responses. Rapid advances in our knowledge of the mechanisms of intracellular signalling has greatly increased understanding of how cells function physiologically, how they malfunction pathologically, and how their behaviour might be manipulated therapeutically.

Introduction The evolution of cellular life was only possible through the development of an insulating barrier to the external world, the plasma membrane, allowing manipulation of the intracellular environment. However, to be able to respond to the extracellular milieu and to each other, primitive cells needed to transmit information across the plasma membrane, leading to the evolution of intracellular signalling processes. The progression to multicellularity appears to have depended on the development of robust and sophisticated signal transduction pathways. These evolved through episodes of gene duplication and subsequent protein sequence divergence which peaked at the time of animal-plant-fungi separation (1000 million years ago) and again after the Cambrian explosion (500 million years ago). Moreover, co-option of proteins originally involved in cell structure and metabolism further contributed to the diversification and development of signalling pathways. The resulting complex nomenclature, originating from diverse sources of biological research (especially the fruit fly, *Drosophila*), can be confusing and alienating for the nonspecialist. Transmitting information across the plasma membrane barrier is achieved in one of three ways: 1. Nuclear receptor signalling (e.g. utilized by steroid hormones) employs lipophilic, membrane-permeable ligands which diffuse through the plasma membrane and directly interact with intracellular receptors to alter gene expression and subsequently cell function. Nuclear receptor signalling is limited by the physical properties of the ligand, the absence of a signal amplification

step (limiting sensitivity), and slow response times (since these depend on de novo protein synthesis). 2. Ion channel activation permits rapid changes in membrane voltage and intracellular ion concentrations. These processes, which underlie nerve conduction and muscle contraction, also mediate signalling events in nonexcitable cells and are discussed elsewhere. 3. Cell surface receptors, in contrast, detect extracellular ligand binding and transmit an intracellular signal to alter cell function. There are seven main solutions to the problem of transmembrane signal transduction that have evolved in mammalian cells. Heterotrimeric G-protein-coupled-receptors (GPCRs), Wnt, and Hedgehog (Hh) signalling pathways all utilize cell surface molecules with seven transmembrane-spanning domains (7TM) which undergo conformational change on ligand binding triggering intracellular signalling cascades. Alternatively, ligand engagement can be sensed through activation (upon receptor aggregation) of intracellular enzyme cascades. This mechanism is utilized in tyrosine kinase (TK)-dependent receptor signalling and in the serine/threonine-dependent signalling of the transforming growth factor beta (TGF- β) receptor superfamily. Receptors have also evolved which, upon ligand-induced aggregation, recruit cytoplasmic molecules into large signalling complexes via homotypic protein domain interactions. These include the TNF α and Fas receptors as well as the Toll-like receptors (TLRs). Finally, Notch signalling employs ligand-dependent receptor cleavage and nuclear translocation of the receptor fragment to induce gene expression changes.

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3.5 Intracellular signalling 257 Principles of receptor signalling Receptor signalling pathways, in transmitting an extracellular message to the cell interior, have to deal with the same fundamental problems of information transmission as other processes, such as electronic systems. These include signalling sensitivity, robustness, resolution, and integration.

Sensitivity The sensitivity of signalling pathways varies enormously and is determined by both activation threshold and signal amplification. The activation threshold for a pathway can be set by (1) the affinity, avidity, and dissociation rates of receptor-ligand interaction, and also (2) the amount of activated intermediary molecules required to propagate the signal. For example, activation of IgG receptors (Fc γ R) is determined by both the density and subclass of IgG coating an antigen (thereby affecting receptor engagement) but also by the level of receptor tyrosine phosphorylation achieved (which is influenced by the balance of receptor-associated tyrosine kinases and phosphatases). Amplification is usually achieved through one or more enzymatic steps in the signalling pathway and permits extremely low levels of stimuli to trigger signal transduction. Examples include the ability of rod photoreceptors to respond to individual photons of light and the successful recognition of individual peptide-bound major histocompatibility complex molecules by T-cell receptors. Very high amplification tends to lead to yes-no binary outputs (a pathway is either 'on' or 'off') as well as low signal-to-noise ratios. In contrast, nonamplified systems permit more fidelity of signal representation (with high signal-to-noise ratios), as illustrated by the response of TGF β superfamily receptors to morphogenic gradients during development. By altering sensitivity (through changes in threshold and amplification), signalling pathways can greatly extend the dynamic range of stimulus intensities they respond to without saturating. This process is known as adaptation.

Signal robustness Robustness refers to the ability of systems to function correctly in the presence of invalid inputs or hostile environments. Robustness in cellular signalling can be enhanced by both positive and negative feedback loops. For example, a pathway which enhances the formation of its own ligand amplifies, stabilizes, and prolongs signalling. Such events are commonly seen in development (where correct signal transmission is critical) but also occur aberrantly in cancer (through the establishment of autocrine signalling loops). Negative feedback

cycles also stabilize fluctuations, rapidly returning signals to pre-excitation levels and thus minimize subthreshold signalling. Another mechanism of increasing signal robustness is the use of parallel, redundant signalling cascades which ensure that interruption of one pathway does not disrupt signal transmission. Signal resolution Signalling pathways need to respond appropriately to temporal and spatial changes in stimulus.

- Temporal resolution is determined by the speed of initiation and termination of signalling and varies from milliseconds (e.g. ion channel activation and GPCR-like sensory transduction), seconds to minutes (as seen in TK-dependent signalling of immunoreceptors), or hours (such as Notch signalling in development). While fast temporal resolution permits rapid detection of changing external environments, slow receptor kinetics will, in effect, average extracellular signals over time, removing fluctuations in signal intensity, and results in improved signal-to-noise ratios.
- Spatial resolution is the ability of cells to detect the localization of a stimulus. It is critical for many processes including cell migration during embryogenesis and inflammation, cell-cell interactions (e.g. immune synapse formation), and phagocytosis. Spatial resolution requires subcellular containment of activated signalling components which can be achieved by: (1) the restriction of lateral diffusion of activated membrane receptors by sphingolipid microdomains or cytoskeletal barriers; (2) the presence of a cordon of inhibitory molecules limiting signal spread (as observed at the leading edge of chemotactic cells where the phosphatase PTEN localizes phosphatidylinositol 3,4,5-triphosphate (PIP3) production); and (3) the sequestration of active signalling proteins within a large multimolecular complex which localizes activity to a specific region of the cell.

Signal integration As with complex neurological systems, individual cells can integrate multiple input signals (through interactions between different signalling pathways) to perform simple Boolean operations (sensing 'signal 1 AND signal 2', 'signal 1 OR signal 2', and 'signal 1 NOT signal 2'). For example, to achieve full activation, T lymphocytes need to receive simultaneous signals from both the T-cell receptor (TCR) and its coreceptor, CD28. They thus identify 'TCR signal AND CD28 signal'. However, if only TCR signalling is triggered (TCR signal NOT CD28 signal), cells respond by becoming unresponsive (anergic) to further stimulation. For certain aspects of T-cell function, however, another coreceptor, ICOS, may substitute for CD28 signalling and leads to full cellular activation (ICOS signal OR CD28 signal). Cells are also able to use various signal transduction topologies to stabilize outputs and integrate information about signal amplitude, duration, frequency and (sometimes) spatial orientation. Examples include threshold detection to allow transformation of graded (analogue) inputs into an all or nothing (digital) output (e.g. cell fate decisions) and negative feedback amplification (where an input is enzymatically amplified and then inhibited by the resultant output) to improve noise and smooth outputs. Cells also undertake more complex signal processing. The recent application of high-throughput genetic manipulation, the identification of protein-protein interactions by mass spectrometry, and the application of bioinformatic analysis has revealed that, far from being a series of linear processes, intracellular signalling is, in reality, a complex, integrated, and interdependent network of signalling pathways. However, within this web of multiple protein-protein interactions and enzymatic cascades, there are clear signalling 'nodes': important molecules where multiple signal inputs converge, which represent points of physiological, and potentially pharmacological, control of cellular responses.

258 SECTION 3 Cell biology Specific signalling pathways The description of signalling pathways here is limited to the seven main types of receptor signal transduction mechanisms used by mammalian cells: GPCR, Wnt, Hh, tyrosine kinase-dependent signalling (using the B-cell receptor as an example), TGF β superfamily receptors, TLRs (as an example of pathways utilizing homotypic

protein domain interactions), and Notch signalling. For each pathway, the main physiological roles, the mechanism by which signalling is initiated and controlled, the pathological consequences of signalling dysfunction, and the potential for therapeutic manipulation are described.

G-protein-coupled receptors

The G-protein-coupled receptors (GPCRs) are a large family of approximately 800 7TM proteins which are involved in virtually all aspects of human biology including sensory transduction (of vision, olfaction, taste, and pain) and signalling by peptide hormones, glycoproteins, neurotransmitters, and chemokines. More than 60% of all marketed drugs (and a large proportion of those in development) target GPCRs. The main signalling pathways are summarized in Fig. 3.5.1. GPCRs constitutively associate with heterotrimeric G proteins which consist of a guanine diphosphate (GDP)-bound α subunit (of which there are 16 types) complexed to a $\beta\gamma$ dimer. Ligand binding to a GPCR ruptures an ionic bond between transmembrane domain (TM)-3 and TM-6 inducing a large cytosolic conformational change which permits binding of a G protein. The $G\alpha$ subunit of the G protein is then able to bind guanosine triphosphate (GTP) instead of GDP and allows $G\alpha$ and $\beta\gamma$ subunits to dissociate and interact with effector enzymes (such as adenylyl cyclase and phospholipase C) and small G proteins. Intrinsic hydrolysis of bound GTP to GDP inactivates $G\alpha$ (thus acting as a molecular stopwatch to limit $G\alpha$ activity) and permit binding to $\beta\gamma$ subunits and re-association with receptors. In parallel, GPCRs also interact, through c-terminal phosphorylation by G-protein-coupled receptor kinases (GRKs), with β -arrestins; molecules that mediate ubiquitination-dependent receptor endocytosis, recruitment of c-Src family tyrosine kinases (such as Hck and Yes), and activation of the ERK MAP kinase signalling pathway. Signalling by β -arrestins mediates receptor desensitization, cellular degranulation, chemotaxis, and cell survival. Many GPCRs have multiple physiological ligands, binding at distinct extracellular sites, which can selectively activate specific intracellular signalling pathways. These 'biased ligands' have driven pharmaceutical efforts to develop compounds, binding orthosterically or allosterically, that might selectively modulate specific intracellular signalling pathways. More recently, *in vitro* studies have shown the feasibility of using membrane-permeable peptides or intrabodies to conformationally alter GPCRs from the cytosolic surface and discretely target specific signalling cascades. As might be expected from their critical role in peptide hormone signal transduction, loss-of-function and gain-of-function mutations of multiple GPCRs and heterotrimeric G proteins have been implicated in both hereditary and sporadic endocrine diseases. Somatic $Gs\alpha$ mutations, which disrupt intrinsic GTPase function resulting in prolonged activation, are found in 40% of growth-hormone-secreting pituitary adenomas as well as McCune-Albright syndrome (bone fibrous dysplasia, endocrinopathy with hormone oversecretion). In contrast, heterozygous inactivating mutations of $Gs\alpha$ result in Albright's hereditary osteodystrophy. Simple loss-of-function mutations in GPCRs usually cause recessive conditions. For example, mutations in the luteinizing hormone (LH) receptor cause autosomal recessive familial hypogonadism. By contrast, gain-of-function mutations may result from changes in: (i) ligand specificity (e.g. mutant FSH receptors responding to hCG in ovarian hyperstimulation syndrome); (ii) ligand sensitivity (e.g. full activation of mutant calcium-sensing receptors at physiological calcium levels in Bartter syndrome type V); (iii) increased basal activity in the absence of ligand (e.g. LH receptor mutations causing male-limited GPCR signalling).

Downstream signalling

Inhibition of $G\alpha$ signalling

Receptor internalization

β -arrestins

P $G\alpha$ $G\alpha$

Fig. 3.5.1 G-protein-coupled receptors (GPCRs). GPCRs constitutively associate with the guanosine diphosphate (GDP)-bound α ($G\alpha$) and $\beta\gamma$ subunits of heterotrimeric G proteins. Ligand binding induces receptor conformational change allowing $G\alpha$ to bind guanosine triphosphate (GTP) which

permits the subunits to dissociate and interact with effector enzymes. GPCRs also interact with β -arrestins, following C terminal phosphorylation by GPCR kinases (GRK), which mediated receptor internalization and other signalling events.

3.5 Intracellular signalling 259 precocious puberty); and (iv) decreased desensitization (e.g. KISS-1 receptor mutations causing precocious puberty). GPCR signalling has also been exploited by pathogens. HIV binding to the chemokine receptor CCR5 mediates cellular invasion and alters immune cell function. *Vibrio cholerae* toxin A1 induces ADP-ribosylation of G α s preventing GTP hydrolysis (resulting in persistent activation and leading to cyclic AMP-driven secretory diarrhoea). *Bordetella pertussis* toxin A freezes G α i in an inactive GDP-bound conformation (through ADP-ribosylation) which prevents phagocyte chemotaxis, bacterial engulfment, and intracellular killing.

Wnt signalling Wnt signalling plays a major role in epidermal, haematopoietic, and neural stem cell development and has been implicated in oncogenesis (particularly of colonic, ovarian, and hepatocellular carcinoma and melanoma) thought to arise through stem cell dysfunction. 19 genes are defined in humans encoding Wnt ligands, a family of secreted, palmitoylated, cysteine-rich proteins which bind to surface receptors (called Frizzled-class proteins) variably complexed to different coreceptors (LRP5, 6; MUSK; PTK7; ROR1, 2; RYK; Syndecan; and Glypican). The name Wnt is derived from Wingless, a *Drosophila* gene and the molecule Int-1 (integration of mammary tumour virus). Canonical Wnt signalling is summarized in Fig. 3.5.2. In the absence of Wnt ligands, newly synthesized β -catenin is complexed within the cytoplasm to two scaffolding proteins, Axin and APC (adenomatous polyposis coli). Serine/threonine phosphorylation of β -catenin, by two proteins GSK3 β and CK1, initiates its ubiquitination and subsequent proteosomal degradation. In the absence of β -catenin, the transcriptional complex Tcf/Lef represses gene expression. Wnt ligands induce coaggregation of LRP 5/6 (an LDL receptor family member) and the 7TM receptor, Frizzled, and results in phosphorylation of the scaffolding protein Dishevelled and sequestration of Axin. The resultant inhibition of the β -catenin destruction complex increases cytoplasmic levels of β -catenin and permits its nuclear translocation and binding to Tcf/Lef to form an activatory transcription complex which triggers gene expression. β -catenin-independent signalling pathways can also be triggered by Wnt signalling. These include (a) planar cell polarity signalling that regulates cell polarity and cytoskeletal rearrangements through activation of the GTPases Rac1 and RhoA and is essential for correct gastrulation, neural tube closure, and orientation of inner ear stereocilia; and (b) Wnt-calcium signalling, involving activation of heterotrimeric G proteins leading to phospholipase C-mediated production of inositol 1,4,5-trisphosphate and subsequent calcium signalling, which influences cell motility and gene expression during tumorigenesis, inflammation, and neurodegeneration. Evidence suggests that both these noncanonical signalling pathways inhibit canonical β -catenin-dependent signalling and are favoured by certain Wnt ligands (Wnt5A, 11) and coreceptors (ROR1 and 2) combinations. The critical role of Wnt signalling in stem cell regulation within intestinal villi underlies its association with colonic malignancies. Raised nuclear β -catenin levels (leading to persistent Wnt-dependent gene expression and eventually malignant transformation) occur in the presence of (1) mutations in either APC (found in most sporadic colorectal cancers as well as familial adenomatous polyposis) or Axin which impair β -catenin binding or (2) activating mutations of β -catenin, preventing its phosphorylation. Therapeutic manipulation of Wnt pathway signalling is currently being investigated. Small molecule agonists to enhance tissue repair and wound healing are under preclinical development. Lithium, at Frizzled X Nucleus LRP P GSK3- β P P CK1 Axin APC Frizzled Nucleus Dsh LRP Axin Wnt Wnt β -catenin β -catenin LEF/ TCF GSK3- β APC LEF/ TCF β -catenin Fig. 3.5.2 Wnt signalling. In the absence of Wnt ligands,

newly synthesized β -catenin is bound by Axin and APC (adenomatous polyposis coli), phosphorylated by GSK3 β and CK1 and consequently degraded by the ubiquitin- proteasome system. Wnt ligands induce coaggregation of the surface receptors Frizzled and LRP, leading to phosphorylation of Dishevelled (Dsh), sequestration of Axin and inhibition of β -catenin degradation. β -catenin can then translocate to the nucleus, bind the transcription complex LEF/TCF and trigger gene expression.

260 SECTION 3 Cell biology least in vitro, enhances canonical Wnt signalling (through inhibition of GSK3 β), which may underlie some of its effects in psychiatric disorders. Antagonists of Wnt pathway signalling are in clinical trials as antitumour agents. These include inhibitors of Porcupine (an acyltransferase which specifically palmitoylates WNTs, enabling secretion), monoclonal antibodies targeting various Frizzled isoforms, and blockers of β -catenin interaction with its transcriptional coactivator CBP. Hedgehog (Hh) signalling has important roles in embryogenesis, tissue repair, and tumorigenesis. Hh proteins are named after the appearance of the embryo in classical *Drosophila* mutants and have been conserved as regulators of development in vertebrates. They act as short- and long-range morphogens (determining cell fate), mitogens (controlling cell proliferation) and as inducing factors (regulating the form of developing organs). There are three human homologues of Hh—Sonic Hh, Indian Hh, and Desert Hh—secreted as lipid-conjugated hydrophobic peptides with distinct patterns of spatial and temporal distribution. In vertebrates, all Hh signalling appears to take place on, and is regulated by, primary cilia. Hedgehog binds to a cell surface receptor, Patched-1, relieving constitutive repression of a (predominantly endosomal) 7TM protein, Smoothened, which is then recruited to the plasma membrane and primary cilium (Fig. 3.5.3). Active Smoothened increases the formation of the activator form of the transcription factor complex, GLI (GLI_A) which stimulates Hh target gene transcription. Control of Hh signalling occurs through (1) constitutive repression of Smoothened; (2) phosphorylation of GLI by other signalling pathways (such as Notch) which generates a repressor transcription complex GLI_R preventing gene expression, and (3) inhibition of nuclear translocation of GLI_A by two cytoplasmic proteins, SUFU and Iguana. In addition, noncanonical (GLI-independent) Hh signalling can occur through Smoothened-independent pathways (for example through Patched-1 dependent regulation of cyclin B1 and consequently apoptosis) and through cytoskeletal regulation and calcium oscillations by Smoothened. Mutations in human Sonic Hh, the best characterized member of these developmental regulatory proteins in mammals, result in developmental disorders such as holoprosencephaly which is frequent in aborted fetuses and characterized by severe malfunctions including cyclopia. Drugs which interfere with sterol synthesis cause such malformations because they interfere with the addition of cholesterol to the N-terminal domain of the Sonic Hedgehog protein after processing, thereby preventing normal trafficking and secretion of the ligand. Uncontrolled Hh signalling appears to promote tumorigenesis. Gorlin syndrome, caused by an inactivating mutation of Patched-1, is characterized by the development of multiple basal cell carcinomas and medulloblastomas. Moreover, most sporadic basal cell carcinomas show evidence of inactivating mutations in Patched-1 or activating mutations in Smoothened, while a proportion of medulloblastomas demonstrate increased Hh signalling (due to inactivating mutations of Patched-1 or SUFU). In addition, a truncated alternative splice variant of GLI1 has been identified in many glioblastomas and breast cancers. Therapeutic disruption of Hh signalling through blocking Smoothened (e.g. vismodegib) and GLI1 (arsenic trioxide) are now licenced as antitumour agents, while Shh inhibition by small molecules or monoclonal antibodies show promising efficacy in vitro and in vivo. Tyrosine kinase-dependent signalling Tyrosine kinases

mediate signalling by several different receptor families including those with receptor-associated TK activity (such as Other signals Patched-1 Smoothed Gli X Patched-1 Smoothed Gli GliA Hedgehog X GliA GliR Iguana SUFU Nucleus Nucleus Endosome Endosome Hedgehog signalling Hedgehog SUFU P Fig. 3.5.3 Hedgehog signalling. Binding of the soluble ligand Hedgehog to its receptor, Patched-1, relieves constitutive repression of the protein Smoothed which now acts on the transcription factor complex Gli to increase the amount of activator form (GliA) relative to repressor form (GliR) and thus promote gene expression. Control of signalling is achieved by inhibition of nuclear translocation of GliA by two cytoplasmic proteins SUFU and Iguana, and phosphorylation of Gli (by several different signalling pathways including Notch) promoting GliR formation.

3.5 Intracellular signalling 261 as epidermal growth factor receptors) and those which recruit soluble TKs to initiate signalling (such as immunoreceptors, integrins, and cytokine receptors). In general, signal transmission is receptor aggregation-dependent, rapid in onset (of the order of seconds to minutes) and, once initiation thresholds are surpassed, greatly amplified (due to multiple enzyme-dependent steps). As expected, dysregulated TK signalling contributes to both oncogenesis and immunodeficiency. The B-cell receptor (BCR) serves as a useful example. The BCR complex consists of a surface immunoglobulin noncovalently associated with Ig α and Ig β subunits, each of which contains a cytoplasmic immunoreceptor tyrosine activation motif (ITAM). Antigen-induced receptor aggregation permits loosely associated c-Src family TKs (such as Lyn and Fyn) to phosphorylate subunit ITAMs which can then strongly bind c-Src family and Syk family tyrosine kinases. A second wave of adaptor molecules (such as BLNK), small G proteins (such as Ras and Rac), and kinases such as phosphatidylinositol-3-kinase (PI-3K) are recruited to the signalling complex. PI-3K generates phosphatidylinositol 3,4,5-triphosphate (PIP3) from the plasma membrane lipid phosphatidylinositol 4,5-triphosphate (PIP2(4,5)). PIP3 recruits cytoplasmic molecules (through their Pleckstrin homology domains). These include: (1) Bruton's tyrosine kinase (BTK), mutation of which result in X-linked agammaglobulinaemia; (2) AKT; and (3) phospholipase C (PLC), which generates inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) from PIP2(4,5), leading to intracellular calcium signalling and protein kinase C (PKC) activation. The fully formed signalling complex can then activate downstream signalling pathways such as ERK, JNK, and p38 MAP kinases, NF κ B, and NFAT (Fig. 3.5.4). Signal transduction is regulated at certain steps including: (1) CD45-dependent dephosphorylation of src family kinases which is necessary to permit ITAM engagement; (2) the activity of the phosphatidylinositol phosphatase, PTEN, which converts PIP3 back to PIP2(4,5) thereby limiting signalling complex formation; and (3) the density of immunoreceptor tyrosine inhibitory motif (ITIM)-containing inhibitory receptors (such as Fc γ RIIb and CD22) associated with the signalling complex. PTEN (phosphatase and tensin homologue) is a human tumour suppressor gene; it is one of the most frequently lost tumour suppressors in cancer and is mutated in both Cowden's and Proteus syndromes. These inhibitory receptors recruit soluble tyrosine phosphatases (such as SHP1) which limit phospho-ITAM generation, inositol phosphatases (such as SHIP) which hydrolyse PIP3 to PIP2(3,4), and inhibitors of small G-protein signalling (such as p62 DOK). Small molecule inhibitors of receptor tyrosine kinases are in clinical use in the treatment of chronic myeloid leukaemia (imatinib), renal cell carcinoma and gastrointestinal stromal tumours (sunitinib). Several strategies have been adopted to disrupt BCR signalling in chronic lymphocytic leukaemia (CLL) where proliferation is driven by aberrant BCR activation, and in autoimmune conditions, where inappropriate, BCR signalling leads to autoantibody production and self-antigen presentation by B cells. Small molecule inhibitors of src-

like tyrosine kinases (dasatinib), BTK (ibrutinib), PI3 kinase δ (idelalisib), and Syk kinase (fostamatinib) are now licensed therapies for CLL, with the latter also increasingly used for autoimmune conditions. Interest has also focused on ways to recruit ITIM-containing inhibitory receptors to the BCR complex to reduce activatory signalling in autoimmunity. Monoclonal antibody therapy targeting the inhibitory receptor CD22 (epratuzumab) and B cell receptor signalling

P P P P P P P P SHP1 Lyn Syk PLC BTK Lyn SHIP ITAM ITIM PKC Fc γ RIIb BCR BLNK
 Calcium signalling Downstream signalling Activation of NFAT, NF κ B Downstream signalling
 Activation of ERK, JNK, & p38 MAP kinases DAG SHIP Ras Rac PTEN PI-3K

Fig. 3.5.4 B-cell receptor (BCR) signalling. Antigen induces BCR aggregation leading to phosphorylation of cytoplasmic ITAMs (immunoreceptor tyrosine activation motifs) and subsequent binding and activation of soluble tyrosine kinases such as Lyn and Syk. A second wave of adaptor molecules (such as BLNK), small G proteins (such as Rac and Ras) and kinases, including phosphatidylinositol 3-kinase (PI-3K) are then recruited to the signalling complex. PI-3K generates phosphatidylinositol 3,4,5-triphosphate (PIP3) from PIP2(4,5) recruiting further molecules including Bruton's tyrosine kinase (BTK) and phospholipase C (PLC). The latter splits PIP2(4,5) into inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG) leading to calcium signalling and protein kinase C activation. Signalling is controlled by coaggregation of inhibitory receptors, such as Fc γ RIIb, which, through their ITIM (immunoreceptor tyrosine inhibitory motifs), recruit and activate tyrosine phosphatases (such as SHP1), limiting ITAM phosphorylation, and the inositol phosphatase SHIP, which together with the phosphatidylinositol phosphatase PTEN, reduce PIP3 levels.

262 SECTION 3 Cell biology promoting its BCR coaggregation and internalization is currently in late stage clinical trials for systemic lupus erythematosus (SLE), while a bi-specific antibody-based molecule is being developed to colligate the inhibitory Fc γ receptor, Fc γ RIIb, with the Ig β subunit of the BCR to block activation in autoimmune conditions. Transforming growth factor beta (TGF- β) superfamily The TGF- β superfamily of about 20 ligands, including TGF- β , activin, nodal, endoglin, bone morphogenetic proteins (BMP), and growth and differentiation factors (GDFs), have important roles in embryogenesis (where they form morphogenic gradients), extracellular matrix (ECM) remodelling and wound healing, and immunoregulation. Pathologically, TGF β overactivity has also been shown to drive epithelial-mesenchymal transition (a process that contributes to cancer progression, neo-intimal hyperplasia, and tissue fibrosis), promote connective tissue disruption in conditions such as Marfan syndrome, and suppress antitumour immunity. Receptors contain cysteine-rich extracellular domains, a single TM domain and an intracellular serine/threonine kinase domain. Ligands (all of which contain three intramolecular disulphide bonds termed a 'cysteine knot') are secreted as inactive homodimers bound within a large latent complex (LLC) which, in the case of TGF β , consists of a latency-associated peptide (LAP) and latent TGF β -binding protein (LTBP). The LLC is extensively bound to extracellular matrix components. Proteolytic cleavage (by matrix metalloproteinases and other enzymes), integrin binding, and pH changes will release active TGF β which can then trigger the aggregation of type I and type II receptor homodimers into heteromeric complexes (Fig. 3.5.5). Phosphorylation of type I receptors (by type II receptor serine/threonine kinases) permits recruitment and subsequent phosphorylation of intracellular signalling molecules called receptor (R-) SMADs. SMADs are homologues of the Caenorhabditis elegans protein SMA and the Drosophila protein Mothers against decapentaplegia. Phosphorylated R-SMADs, in turn, bind the key regulator, SMAD4. The R-SMAD/SMAD4 complex translocates to the nucleus where, after associating with other cofactors, it regulates gene transcription. Inhibition of signalling is achieved through transcriptional induction of inhibitory (I-) SMADs, which

competitively bind type 1 receptors (preventing SMAD complex formation) and target receptors for ubiquitin-dependent degradation, and prevention of nuclear translocation of R-SMAD/SMAD4 via phosphorylation by ERK, MAPK, and CDK kinases. Noncanonical signalling involves activation by the heteromeric receptor complex of a series of pathways including: TNF receptor-associated factor (TRAF) 4, TRAF6, TGF β -activated kinase (TAK)-1, MAP kinase, PI3 kinase- AKT, and NF- κ B. Defective signalling of TGF β superfamily pathways has been implicated in Camurati-Engelmann disease (a progressive diaphyseal dysplasia affecting long bones), oncogenesis (particularly skin cancers), several fibrotic conditions (including systemic sclerosis), familial primary pulmonary hypertension (BMP receptor 2 Nucleus TGF β P P Type I Type II R-SMADs R-SMADs SMAD-4 R-SMADs SMAD-4 R-SMADs SMAD-4 I-SMADs P P P ERK MAPK CDK Inactive TGF β LLC Fig. 3.5.5 TGF β signalling. TGF β is secreted in as an inactive dimer bound to a latency-associated peptide (LAP) and a latent TGF β binding protein (LTBP) to form a large latent complex (LLC). Following release from the extracellular matrix and proteolytic cleavage, active TGF β binding induces heteromeric receptor complexes leading to phosphorylation of type I receptor cytoplasmic tails permitting recruitment and activation of R-(receptor) SMADs, which in turn bind SMAD-4. The R-SMAD/SMAD4 complex then translocates to the nucleus where it interacts with other cofactors to control gene expression. Inhibition of signalling is achieved by transcriptional induction of inhibitory SMADs (I-SMADs) which prevent R-SMAD/SMAD4 complex formation and target receptors for degradation. Negative regulation of R-SMAD/SMAD4 nuclear accumulation and transcriptional activation is achieved through serine/threonine phosphorylation through ERK, MAPK, and CDK kinases.

3.5 Intracellular signalling 263 mutations), and hereditary haemorrhagic telangiectasia (endoglin mutations). Multiple strategies for therapeutic regulation of TGF β signalling in cancer and fibrosis are currently undergoing clinical and preclinical evaluation, including antisense oligonucleotide and antisense RNA blocking ligand synthesis, ligand traps (e.g. soluble Fc-receptor fusion proteins to reduce active ligand concentrations), inhibitors of ligand activation, monoclonal antibodies targeting either ligands or receptors, and small molecule inhibitors of receptor signalling. Pirfenidone, an antifibrotic drug which probably acts by reducing TGF β activity, has recently been approved for use in idiopathic pulmonary fibrosis. Toll-like receptor signalling Several signalling pathways utilize protein-protein binding via homotypic domain interaction. These include TNF α receptor signalling, which uses death domains (DD), caspase signalling (utilizing CARD domains), and TLR signalling (which uses DD and Toll/interleukin 1 (TIR) domain interactions). I have focused on TLR signalling as an example. The nine types of mammalian TLR are found on both the cell surface (TLR1,2,4,5,6,) and within endosomal compartments (TLR3,7,8,9) and recognize distinct microbial products (as well as some endogenous ligands). They direct the innate immune response against pathogens, triggering inflammatory and antiviral mediator release. In addition, TLR-induced maturation of dendritic cells permits processing and surface presentation of internalized antigen, resulting in stimulation of cognate T cells and induction of adaptive immunity. In the case of TLR4 (summarized in Fig. 3.5.6), engagement of lipopolysaccharide (LPS) triggers receptor aggregation and conformational change which recruits cytoplasmic adaptor proteins (MyD88, MAL, TRIF, and TRAM) through TIR domain interactions. MyD88 in turn, through DD interactions, recruits and activates the serine/threonine kinases IRAKs which mediate ubiquitination-dependent formation of a large oligomeric signalling complex (the 'signalosome') which permits activation of NF κ B (generating production of pro-inflammatory cytokines such as TNF α). TRIF, in contrast, activates interferon-regulatory factors (IRFs) which trigger the generation of IFN α and β (which are crucial to antiviral host immunity). Regulation of signalling occurs at several levels including: (1) reduced

membrane recruitment of MyD88; (2) disruption of IRAK signalling by the inhibitory molecule IRAK-M; and (3) inhibition of TRIF signalling by the cytoplasmic protein SARM. Polymorphisms in components of TLR signalling have been associated with increased susceptibility to Gram-negative infections and septic shock (TLR4), Gram-positive infections (TLR2, IRAK4, TLR-4 signalling LPS Nucleus IRAK-M SARM MAL MyD88 TRIF TRAM LPS LPS IRAK IFN α IFN β TNF α TLR4 NF κ B NF κ B IRFs IRFs MD2 Fig. 3.5.6 Toll-like receptor signalling. Lipopolysaccharide (LPS) binding to TLR4 triggers receptor aggregation and conformational change recruiting cytoplasmic adaptor molecules (MyD88, MAL, TRIF, and TRAM) through homotypic TIR (Toll/interleukin 1) domain interactions. MyD88 recruits and activates the serine/threonine kinases IRAKs (through Death domain interactions) which in turn activate the nuclear transcription factor NF κ B and switch on transcription of pro-inflammatory cytokines such as TNF α . In contrast, TRIF activates interferon-regulatory factors (IRFs) which trigger generation of type 1 interferon (IFN α and β). Signal regulation is achieved at several levels including inhibition of signalling through IRAK and TRIF by IRAK-M and SARM, respectively.

264 SECTION 3 Cell biology MAL), and tuberculosis (TLR2, MAL). TLR polymorphisms have also been implicated in the development of atherosclerosis. There has been growing interest in therapeutic manipulation of TLR signalling for a variety of conditions. Synthetic TLR agonists are in development or already licenced as vaccine adjuvants (e.g. the TLR4 activator monophosphoryl lipid A), as antiviral therapies (e.g. the TLR7 agonist imiquimod), as antitumour agents (e.g. CpG based oligonucleotides stimulating TLR9, imiquimod), as antiallergy therapy (e.g. TLR7 agonists for asthma). TLR antagonists are being developed for acute and chronic inflammation (targeting TLR2 and TLR4), for sepsis (targeting TLR4), for autoimmunity (e.g. Poly TLR antagonists for SLE), and for specific diffuse B-cell lymphomas containing MyD88 oncogenic mutations (blocking TLR7,8,9 activation). Notch Named after a *Drosophila* protein mutation resulting in a 'notched' wing phenotype, Notch signalling pathways are widely conserved across species and have roles in embryonic development (particularly binary cell fate decisions and terminal differentiation), maintenance of stem cells, and lymphocyte differentiation and signalling. Four Notch receptors (Notch 1 to 4) and five canonical ligands (jagged1, jagged2, Delta-like 1, 3, and 4) have been identified (as well as several noncanonical ligands including contactin). Although synthesized as a single polypeptide, surface Notch receptors are heterodimers consisting of an extracellular region non-covalently linked to a transmembrane/intracellular portion. As shown in Fig. 3.5.7, ligand binding permits extracellular cleavage of Notch heterodimers by TACE (TNF α converting enzyme), an ADAM protease. This allows ubiquitin-dependent endocytosis of Notch. Subsequent cleavage by an endosomal γ -secretase (presenilin) releases the intracellular fragment of Notch which can then associate with the nuclear transcription factor CSL, switching on gene expression (particularly of the HES family of transcription factors). In addition, Notch may also signal through CSL-independent nuclear and cytoplasmic pathways (although incompletely understood) that may be independent of receptor cleavage or occur via cross-talk with NF κ B, TGF β , and hypoxia-induced signalling pathways. Notch signalling is exquisitely sensitive to quantitative changes in receptor and/or ligand concentration, since transduction does not involve enzymatic amplification, and is consequently linear. For example, haplo-insufficiency of Notch 2 leads to Alagille's syndrome, while activating Notch 2 mutations result in diffuse large cell lymphomas. Notch signalling can be physiologically regulated by: (a) alteration in receptor fucosylation regulated by Fringe proteins (which alters ligand specificity); (b) rerouting of intracellular Notch fragments for lysosomal degradation via ubiquitination within multivesicular bodies (which limits intracellular signalling);

and (c) via inhibitory interactions of ligands with receptors when both are expressed on the same cell. These negative cis interactions are critical for the formation of sharp boundaries and lateral inhibition patterns during development. Mutations in Notch pathway proteins have been identified in developmental disorders such as congenital aortic valve disease (Notch 1), neurovascular syndromes such as cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL; Notch 3 mutations) and T-cell acute lymphocytic leukaemia (50% of which have activating mutations of Notch 1 caused either by chromosomal translocation or viral promoter integration). Therapeutic manipulation of Notch signalling has so far focused on γ -secretase inhibitors, which have shown promise as antitumour agents in preclinical studies and early phase clinical trials. Nucleus Endosome TACE GSK3- β Notch γ -secretase CSL Notch ligand cis inhibition Lysosomal degradation Fig. 3.5.7 Notch signalling. Ligand binding permits extracellular cleavage of surface Notch receptors (which form as heterodimers) by an ADAM protease TACE which allows ubiquitin-mediated internalization of membrane associated receptor fragment. Further cleavage by an endosomal γ secretase releases a cytoplasmic fragment which associates with the nuclear transcription factor CSL and drives gene expression. Negative signal regulation can occur through lysosomal degradation of internalized receptor fragments or of 'cis' receptor-ligand complexes.

3.5 Intracellular signalling 265 Conclusion The transmission of information across the plasma membrane is achieved through a limited number of types of signalling pathway. Recent progress in defining the mechanisms of signal transduction ('learning the language of intracellular communication') has permitted huge advances in our understanding of disease pathophysiology. The last decade has seen an explosion of new therapies aimed at manipulating signalling therapeutically, with the promise of accelerated future drug discovery with improved molecular characterization of key nodal signalling events. FURTHER READING Akhurst RJ, Hata A (2012). Targeting the TGF β signalling pathway in disease. *Nat Rev Drug Discov*, 11, 791–811. Barolo S, Posakony JW (2007). Three habits of highly effective signaling pathways: principles of transcriptional control by developmental cell signaling. *Genes Dev*, 16, 1167–81. Call ME, Wucherpennig KW (2007). Common themes in the assembly and architecture of activating immune receptors. *Nat Rev Immunol*, 7, 841–50. Clevers H (2006). Wnt/ β -Catenin signaling in development and disease. *Cell*, 127, 469–80. Cook DN, Pisetsky DS, Schwartz DA (2004). Toll-like receptors in the pathogenesis of human disease. *Nat Immunol*, 5, 975–9. Dorner T, et al. (2015). The mechanistic impact of CD22 engagement with epratuzumab on B cell function: implications for the treatment of systemic lupus erythematosus. *Autoimmun Rev*, 14, 1079–86. Fiúza U-M, Arias AM (2007). Cell and molecular biology of Notch. *J Endocrinol*, 194, 459–74. Goetz SC, Anderson KV (2010). The primary cilium: a signalling centre during vertebrate development. *Nat Rev Genet*, 11, 331–44. Guruharsha KG, Kankel MW, Artavanis-Tsakonas S (2012). The Notch signalling system: recent insights into the complexity of a conserved pathway. *Nat Rev Genet*, 13, 654–66. Hennessy EJ, Parker AE, O'Neill LAJ (2010). Targeting Toll-like receptors: emerging therapeutics. *Nat Rev Drug Discov*, 9, 293–307. Ingham PW, McMahon AP (2007). Hedgehog signalling in animal development: paradigms and principles. *Genes Dev*, 15, 3059–87. Kahn M (2014). Can we safely target the WNT pathway? *Nat Rev Drug Discov*, 13, 513–32. Kanzler H, et al. (2007). Therapeutic targeting of innate immunity with Toll-like receptor agonist and antagonists. *Nat Med*, 13, 552–9. Lefkowitz RJ, Shenoy SK (2005). Transduction of receptor signals by β -arrestins. *Science*, 308, 512–17. Milligan G, Kostenis E (2006). Heterotrimeric G-proteins: a short history. *Br J Pharmacol*, 147, S46–55. National Cancer Institute/Nature. Pathway Interaction Database. <https://wiki.nci.nih.gov/pages/viewpage.action?pageId=315491760> National Center for

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